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THE RISE OF SAP IN TALL GRAPEVINES[1,2]

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The common northern grapevine (*Vitis labrusca* L.) grows rather abundantly in the wooded area around Woods Hole, Massachusetts. It frequently grows as a liana, climbing high in various trees, and attaining heights of 17 to 18 meters. In the winter the vessels of the stem and branches are filled with gas and usually contain no sap. With the warm weather in April and May, and before the leaves are out, the vines become full of sap which drips out from the slightest cut in the wood of stem or twigs. The vessels are so wide and so closely packed together that one can see the light through a piece of vine 2.5 cm long, and one can easily blow air through a meter-long section. The sap will flow easily back and forth in a section when it is slightly tilted.

This material seemed suitable for measurements of sap pressure and sap flow. As vines could be found that were taller than ten meters, such measurements possibly could give some direct information on how sap reaches the top of tall trees.

The cohesion theory of Dixon and Joly (14) and Askenasy (4) (cf. Dixon, 13) is now rather generally accepted as the explanation of this phenomenon and it seems well documented as a factor in sap transport in low plants. In its application to tall trees, however, the theory is still disturbingly void of direct evidence. Excellent reviews of sap transport are to be found in the monographs by Crafts, Currier and Stocking (11), and Preston (32). We shall only briefly mention those observations which bear most directly on our subject.

EVIDENCE FOR COHESION IN VESSELS: That a slight tension can exist in the vessels of living plants has long been known. When a twig is cut under water and then sealed air-tight to the top of a water-filled mercury manometer, transpiration will sometimes pull the mercury a few decimeters above barometric level (8, 28, 44, 45, 47).

A twig forced to take in water from a potometer via an artificially produced resistance will often pull the water through at a rate much higher than a vacuum pump can pull it through the same resistance. From this, the pressure drop across the resistance can be calculated to reach as much as 10 to 20 atmospheres, or even more (6, 18, 20, 21, 27, 29, 30, 35, 38). Various other potometer experiments have been performed on undetached branches or stems of trees and bushes (20, 30, 36, 38), which sometimes gave a tension of several atmospheres. As will be shown, however, these experiments did not necessarily mean that the vessels were involved. Kramer (22) found that the transpiration rate in intact tomato and sunflower plants was more than 20 times greater than the rate at which a vacuum pump could pull sap up through the stump. Bode (6) found that, when he wilted shoots of Syringa by closing the cut end with wax, the decrease in diameter of the twig and individual vessels was greater than could be effected by a vacuum pump.

Crafts (10) produced a break in the sap in the intact vessels of wilted Ribes plants by lightly tapping the vessels. The ends of the broken sap columns retracted rapidly, indicating cohesion before the break, provided no air entered. A study of the injection rate of dye solutions into the vessels of various trees led Preston (32) to estimate that cohesion of up to three atmospheres may exist in at least some vessels.

Arcichovskij and his collaborators (1, 2, 3) determined the concentration of sucrose which would neither gain nor lose volume when in contact with the exposed cambium. This was done by a potometer or by observing the absence of optical streaking (Schlieren). The results reflected beautifully the diurnal transpiration cycle and showed also a correlation with height. It was assumed that the osmotic pressure of the test solution equalled the sap pressure in the xylem. A birch tree had, accordingly, sap tensions as high as almost -37 atmospheres and a desert tree gave the staggering figure of -143 atmospheres. The pressure gradient in a birch tree was as high as 5 atmospheres per meter and in the desert tree no less than 44 atmospheres per meter. Whatever these very high figures mean, they are hardly conclusive as to the sap pressure in the vessels, for they were also obtained on excised material where the vessels were open. Dixon (12) estimated that a pressure gradient of the order of 0.2 atmosphere per meter would suffice for normal sap ascent.

Several observations have been used in support of the cohesion theory, which fit it but do not prove its validity. The fact that the cohesion of sap or water in glass tubes or fern annuli (12, 37, 46) may be great enough to overcome both the weight and the esti-

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mated friction of continuous sap columns in the tallest trees is comforting, but does not of course prove the existence of such columns. MacDougal's dendrographic measurements (23, 25) demonstrated a decrease of stem diameter with high transpiration and low water supply, but were never pressure calibrated.

DIFFICULTIES WITH THE COHESION THEORY: There is one situation that applies singularly to tall trees and vines where the cohesion theory gives little comfort. This pertains to the stability, or better the vulnerability, of such a system under tension. Why do not wind storms break the postulated tensile sap strings connecting the leaves with the roots in tall trees and vines, which sometimes reach as high as 100 meters? Great delicacy is required in the laboratory to produce, let alone handle, tensions many times smaller. If these vascular liquid strands broke above a height of 10 to 15 meters, how could they repair? These are old and sound questions (9, 16, 31, 33).

Gases are practically insoluble in ice. When water freezes, dissolved gas is forced out as bubbles (40). If there is sap in the vessels of tall northern trees in the winter, it would freeze, and gas bubbles would be set free inside the vessels, rupturing the sap columns. On the other hand, many tall trees, like our grapevine, may have gas-filled vessels in the winter. In either case, how are the columns reestablished in the spring? Only the grapevine and a few hardwood trees are known to have enough root pressure to do this.

In several species of tall trees air has been found to enter the wet vessels of cut-off twigs at a pressure difference of less than one atmosphere (17, 19, 42). Bailey (5) showed that air leaked through wet border pits of very tall conifers at a pressure of 3 atmospheres, and the "Rückstoss" which Renner (35) and Bode (6) found in potometer experiments was explained as air rushing into the twig when the suction was relieved by decapitation. In these cases, therefore, the end walls of the vessels would let gas through, and would hence be unable to prevent an accidental cohesion break from spreading. Ursprung (45) found that leafy twigs of locust trees (Robinia) wilt if forced to absorb water which is kept at reduced pressure. Observations such as these are, of course, difficult to bring into accord with the classical cohesion theory, and it is not clear by what criteria they can be dismissed, unless it be, as Priestley remarked, because of the difficulty looming ahead.

We describe below a series of observations on the hydraulic situation in the vessels of tall grapevines. Some are difficult to reconcile with the cohesion theory, and some are even difficult to reconcile with each other.

FIG. 1 (*left*). Manometer clamp for measurement of sap pressure. A, manometer; B, head of punch; C, indicator; D, contact button with hollow ground punch protruding.

FIG. 2 (*right*). Manometer clamp on a grapevine.

METHOD FOR ESTIMATING THE VESSEL PRESSURE IN THE GRAPEVINE

The stems of the grapevines used for the measurements were usually 3 to 5 cm in diameter at the base. The sap-conducting vessels are found from the surface of the xylem to a depth of some 3 to 7 mm, according to the size of the vine. The vessels are close together and very large, 0.2 to 0.4 (0.5) mm in diameter.

An instrument was made consisting of a heavy screw-clamp carrying at the head end a vacuum-tight hole-punch connected with a capillary manometer (fig 1). The punch itself is a 1.6 mm ($\frac{1}{16}$") stainless steel rod with a hollow ground cutting edge. A fine bore leads from the punch hole to the manometer tube. For pressures below one atmosphere a fine bore (ca 0.1 mm) thermometer tube is used, containing an indicator drop of boiled Sudan-dyed kerosene. For measurements of pressures above one atmosphere a somewhat bigger bore manometer tube is used (0.5 to 0.2 mm), as in this case sap flows into the manometer tube.

PROCEDURE: The outer bark is stripped off and a thin shaving is removed exposing the phloem. Onto this bare spot a piece of pliofilm is stuck with a little saliva. The manometer clamp is fastened onto the vine (fig 2), with the contact button pressing upon the pliofilm. The screw is tightened, compressing the phloem and the cambium, but not the xylem, and making a vacuum-tight seal.

The punch is given light taps with a hammer until it has advanced about 1 mm. It is then retracted and the manometer is read. The punch is advanced another millimeter and the manometer read again, and so on. Immediately upon removal of the instrument the punch hole is plugged with plasticine, or with a screw hook bearing a rubber gasket.

INTERPRETATION OF THE MEASUREMENTS: A great many measurements have been made by various authors who have applied manometers to bore holes in tree trunks (7, 23, 24, 25, 26, 34, 41). Most investigators are keenly aware of the limitations of this approach, inasmuch as the "stem pressure" may be the resultant of several factors besides the sap or vessel pressure, such as the gas pressure in the trunk and wound exudation.

Our punch cuts a circular hole in the xylem. No chips are formed, as the bottom is merely pushed in. A hole 1 mm deep severs 8 to 10 vessels, and deeper holes proportionally more. All severed vessels intercommunicate. Under the binocular the fresh conducting part of the xylem is semi-transparent and completely permeated with water. When it dries it whitens as the air penetrates the finer structures. There can be little doubt that the instrument gives information pertaining only to vascular pressures. These register immediately and cannot be confused with very slow wound exudations. When healthy vines, cut under water, were given a methylene blue solution practically every vessel in the outer half centimeter of the xylem was stained, as were also the structures between them.

The dead space air of the instrument is 10 mm^3. At pressures below one atmosphere part of this air will enter the vessels. This does not seem to change the results materially, for if one fills the dead space and manometer bore with water the readings are much the same (fig 7). The instrument is weakest in its application to very low pressures and cannot, of course, measure negative pressures because the punch will produce a water vapor break of tensile columns. If this happens, it should record nearly water vapor pressure (0.025 atm). If, therefore, we had a tall hydrostatic cohesion system registering one atmosphere at the base, the pressure would decrease by 0.1 atmosphere per meter of height and would be minus one atmosphere 20 meters up. The instrument would record this hydrostatic gradient only up to a level of 9.7 m. Here the column would be ruptured by the act of measurement, and water vapor pressure would be indicated at any additional height. The measurements would, of course, be higher if vessels containing gas were also severed. The amount of interstitial water in the severed area could add no more than 0.01 atm air pressure to the evacuated vessels.

When the instrument is applied to a grapevine, it is often seen that the minimum pressure is reached in jerks. It is as if water-air menisci were passing valves. Sometimes the minimum pressure oscillates in jerks. These phenomena may be artifacts produced by severing the vessels.

We believe that the measurements in some situations give the sap pressure in the vessels quite accurately. In other situations they require supplementary information for their interpretation. These measurements are offered, in spite of obvious weaknesses, as direct observational approximations where none exist so far, and it is hoped that they will serve as a challenge to promote studies on pressure relations in vessels and finer structures by more refined methods. Upon proper gradient measurement hinges the ultimate clarification of how sap rises in tall plants.

OBSERVATIONS, EXPERIMENTS AND RESULTS

THE VINE STEM AS A WATER CONDUCTOR: If a 10-m section of vine is suspended vertically and filled with water from a long hose hanging beside it and connected to its base, we see that water starts to flow over the cut upper surface of the vine as soon as the water meniscus in the hose is elevated above it. By changing the level of the hose meniscus the water can be made to recede or overflow on the vine side. In these experiments, therefore, the stem behaves like a simple water-filled pipe, where the liquid is movable and exerts a normal hydrostatic pressure.

A tall vine was cut off at the base under water and was left consuming water from a bucket for half an hour. When it was severed at 10 m, 23 ml of water ran out from the base. Another cut 3 m lower produced 27 ml water from the base. Two more sections 60 cm long produced 20 and 24 ml from the base. Shorter pieces yielded less water. This was taken to mean that at every cut the severed water columns ran downwards until the upper menisci were stopped by a "valve" which would freely pass water but not an air-water interface. These valves are spaced approximately 60 cm apart and are very likely the pits in the end walls of the vessels. By turning the vine stem upside down it can be shown that the valves are effective in either direction of flow.

If one takes such a stem section, a meter long or more, which contains sap hanging on the valves, one may nevertheless easily blow air through it, and this will not dislodge the water. Therefore, there are also in the stem many vessels without valves or with valves very much farther apart than the regular distance of 60 cm.

The water consumption was measured in a vine cut off at the base. It was then given mercury. For several minutes this ascended at the same rate as the water, but finally it stopped, having plugged, for a distance of some 50 to 60 cm, all the vessels which took part in the water transport. When the vine was

placed back in water the water consumption was reduced to some 10 %, showing that the resistance in the fine structure for such a distance and with all vessels plugged was considerable. If the plugs were staggered, it could presumably be much less. It was found by Elfving (15) and others that when the vessels of several woody plants were plugged with cocoa butter the water absorption rate was cut down very drastically, proving that the major pathway of the transpiration stream runs through the vessels in these plants also.

Seasonal Variations in Sap Pressure: In the winter the vines are dry, i.e., the vessels are full of air, from the ground to the finest twigs. No water runs out when a meter-long section is cut with two rapid slashes of a machete. During the rest of the year sap can be poured out of either end of such a section, a procedure sometimes used with other vines in the tropics by thirsty travellers.

From the latter part of April to the end of May or the beginning of June, before the leaves are out, pressures of more than one atmosphere obtain in the vines. A series of measurements at ground level is presented in figure 3. In every case it was found that the sap pressure sufficed to support a column of water considerably higher than the top of the vine, sometimes as high as 40 m. There is no correlation between the height of the vine and the pressure found. A horizontal vine, blown down with its host tree several years earlier, showed the same pressure as upright vines. If a little twig is cut, the wood bleeds copiously, showing that the air which filled the vine during the winter has somehow disappeared. Cutting off a new shoot with leaves partly expanded produced no bleeding, and hence there were no direct communications by open vessels between the shoot and the twig xylem at this time.

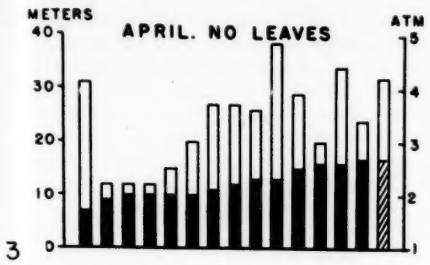
In the summer the pressure in the vessels varies greatly with transpiration. At ground level on a warm, sunny day it was often found as low as 0.1 to 0.2 atm. During and immediately after heavy rain it might rise to slightly above one atmosphere (fig 4). We never observed that the pressure at ground level rose to what would correspond to the height of the vine. In other words, if air had got into some of the vessels by accident, the "root pressure" would not have been able to compress it to a pressure above atmospheric, as would be required for its resolution in the sap. However, our spring-time measurements often showed pressures of the required magnitude. After the leaves have fallen, the vines gradually lose their water and fill with gas at a pressure of one atmosphere. At sap-rising time in the spring this gas again disappears. Hence the vessels are, at least part of the year, pervious to gas.

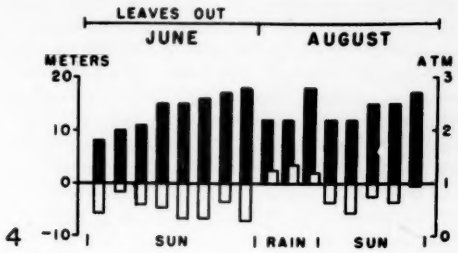
Pressures at Different Heights, *Manometer Measurements:* When pressure readings are taken at different heights in spring before the leaves are out, one obtains quite precisely a hydrostatic gradient, where the pressure is lowered 0.1 atm for every meter's ascent (fig 5). This means that the water in the vessels of the vine at this time exists as continuous water columns. The pressure for a given level is constant around the vine and for different depths in the xylem. Long horizontal vines at this time show no gradient. The vine behaves as a simple water-filled hose.

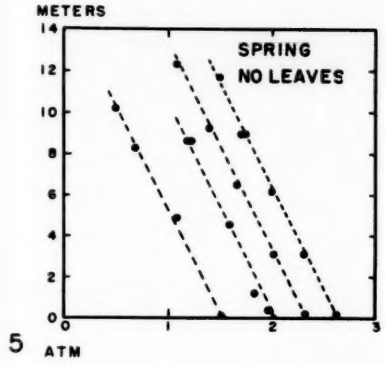
In the summertime when the leaves are out there is *at night* a hydrostatic gradient in the lower part of the vine, just as in the spring, but the pressure is generally lower. *In the daytime* with full transpiration, the picture changes drastically (figs 6 and 7). Figure 8 shows what seems to be a typical pressure profile obtained with the present technique in a tall grapevine. At each level on the vine the punch was driven approximately 1, 2 and 4 mm deep into the xylem. The intermediate depth often gave the lowest reading. Only vines which had all leaves concentrated near the top were used. The main features of these data are: (1) the absence of a hydrostatic gradient, with pressures often slightly increasing at the highest levels, and (2) a great spread of data at each level according to where on the circumference the puncture was made and how deep. The same relation was found in a vine after heavy rain, when naturally all pressures were greater (fig 9). Here the pressures at the base were conspicuously less than higher up. The same general profile was also obtained from a horizontal vine, blown down together with its host tree several years earlier (fig 10). The hydrostatic gradient at night is probably caused by the rise of sap from the roots and possibly also by a gradual filling-up of the vessels which had been evacuated by the transpiration in the daytime. There is good evidence from our data that the pressure profile moves towards increasing pressure during the night.

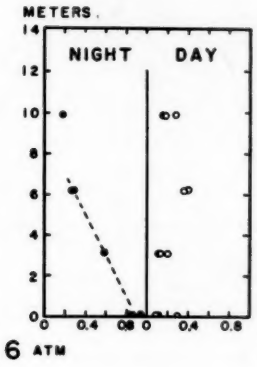
Mercury Injection: A series of experiments was performed by making a sidecut in the intact and moistened stem under mercury. The mercury rose in the vessels to heights of 50 to 60 cm both when the cut was made high above ground and at ground level. Even if cohesion were broken we should expect the mercury to rise to some 70 cm. When, however, the injection was made in the descending shank of a vine which grew in the form of a "U," we found that most columns stopped 80 cm from the cut, even though this was at a lower level than that of the cut. Only a few strands crossed over to the ascending part of the vine. It is clear from this that mercury injection could only faithfully record pressures provided cohesion existed and was maintained at the mercury-water interface, thereby lifting the mercury above barometric level. This did not happen in our experiments. Ursprung (46) found that an oak branch cut under water was unable to draw mercury higher than 60 cm. This again is inconclusive.

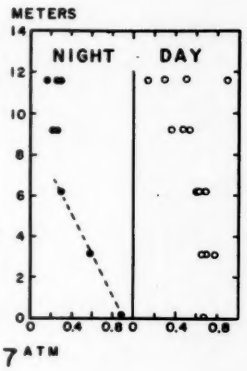
Sap Withdrawal: The pressure measurements gave evidence for positive pressure in the vessels both near the top and at the base of the vine. If this pertained to the sap in the vessels, one should be able to pull sap out by some vacuum technique. The manometer punch was therefore fitted with a capil-

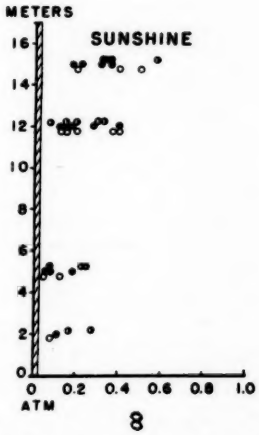


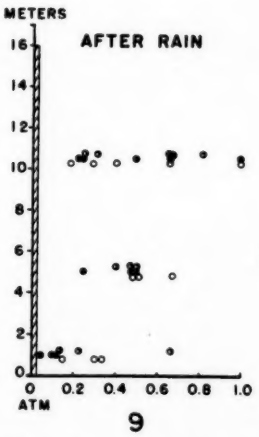












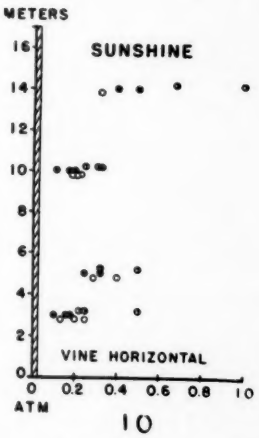


FIG. 3. Sap pressure in 15 grapevines before the leaves were out. Black columns show the height of the vine. The height of the white columns from the base line is the sap pressure in atmospheres. The shaded column is a vine lying horizontally.

FIG. 4. Sap pressure in transpiring grapevines. Black columns are the height of the vine. White columns are the sap pressure in atmospheres.

FIG. 5. Sap pressure in 4 grapevines before leaves are out. Broken lines represent a hydrostatic gradient of 0.1 atm/m.

FIGS. 6 and 7. Diurnal variation in vessel pressure in 2 vines. Broken line is the hydrostatic gradient (0.1 atm/m).

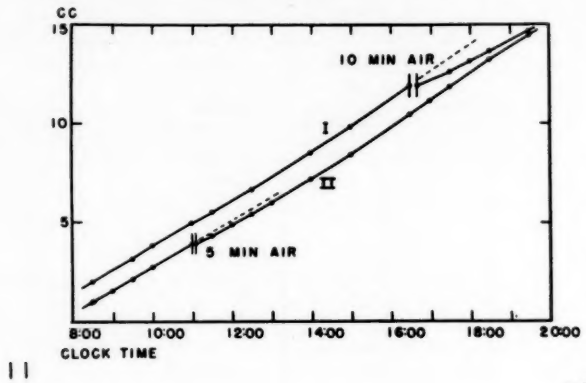
FIGS. 8 to 10. Vessel pressure of tall vines taken at various levels above ground. The shaded column gives the height of the vine. The width of the shaded area represents the water vapor tension. At each level several measurements were taken and for each puncture readings were made at three depths, 1 mm (half-filled circle), 2 mm (black circle), 4 to 5 mm (open circle).

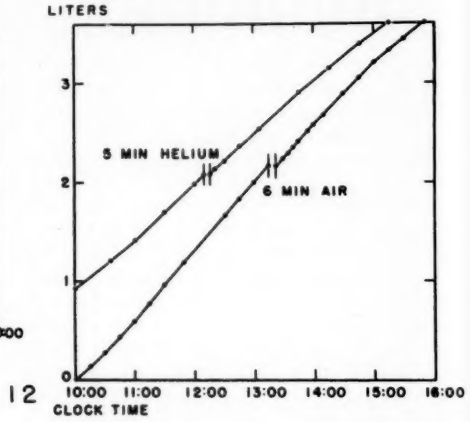
lary T-stud between the punch and the manometer tube. Through the T-connection samples could be drawn through the punch hole in the vine, at the same time as the pressure was read. Vacuum was produced either by a large syringe or by a mercury-filled burette connected with a leveling bulb. In the evening, or at night, when the pressures were near to atmospheric and the hydrostatic condition obtained in the vines, 5 ml samples of sap could easily be withdrawn. At pressures less than half an atmosphere the extraction became difficult. In the daytime we were never able to extract any sap at any level from the vine. On the contrary, the gas-extracted water which filled the dead space of the system was avidly taken up by the vine, and could not be recovered. Very small amounts of gas were sometimes extracted from the vine.

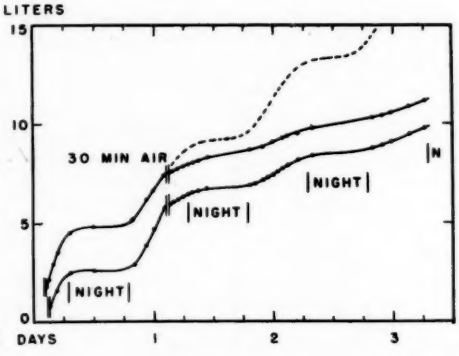
EXPERIMENTS ON WATER UPTAKE IN VINES: One may rightly wonder how tall trees could possibly repair accidental or annually recurring breaks in their water columns. We have, therefore, conducted some simple experiments to find out to what extent air in the conductive system would damage the plant.

Branches with Air Emboli: Two leafy branches of *Vitis labrusca* were cut under water and placed indoors with their bases in test tubes. One was given a 5-min air embolus by lowering the tube. A slight drop in the water absorption rate was soon overcome. Later the other branch was a given a 10-min air embolus. This produced a more marked effect, which was not quite repaired when the experiment terminated (fig 11).

Tall Vines with Air and Helium Emboli: A vine 17 m tall, with the lowest leaves at 13 m, was cut off







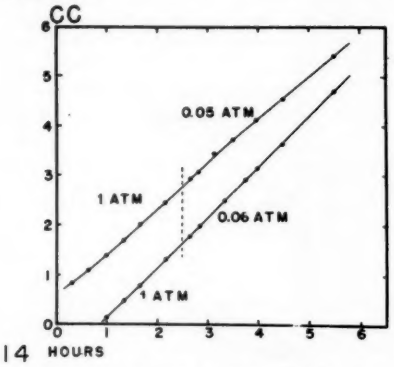


FIG. 11. Water consumption rates of two detached grapevine branches run indoors simultaneously. Between the double lines the vines aspirated air instead of water.

FIG. 12. Water consumption rates of two tall grapevines, the helium-vine 15 m, the air-vine 17 m. Between the double lines the vines aspirated helium or air instead of distilled water. The runs were made simultaneously on a warm, sunny day.

FIG. 13. Water consumption rates of two tall grapevines. The heights were: upper curve, 17 m; lower curve, 12 m. At the first double lines the vines were cut in air. At the second double lines they were given 30 min air, and started to wilt already after 15 to 20 minutes. The broken line is the extrapolated rate if no air had been given.

FIG. 14. Water uptake in two leafy grapevine shoots at atmospheric and reduced pressures.

at the base under water. The cut surface was trimmed until no more gummy material exuded from the central parts. The stump, always held under water, was maneuvered into a milk bottle. This was tied to the vine, and by filling it to the top with distilled water at regular intervals the water uptake rate could be measured.

The vine consumed water at a rate of 10 ml per minute. At noon the bottle was lowered from the vine, giving it 6 minutes of air instead of water, i.e., 60 ml aspirated air. It was established on other vines that the rate of air, mercury, or mineral oil intake by this procedure equals the rate of water intake for several minutes. When the water is removed, therefore, every water column involved in the transpiration recedes up the vine at the normal rate and is followed by air. If the air embolus or emboli, estimated as initially a meter or two long, remained in the vessels and became trapped at the leaves, the weight of the water columns at the lower end of the air blister would expand it to 5 to 6 meters. The water could then rise only to a height of 7 to 8 m, and the water consumption should be drastically reduced. The expected did not happen, however. Water uptake continued without even slowing down (fig 12).

Another vine, 15 m high, with leaves down to 12 m, was given a 5-min helium embolus. The outcome was the same. Even this considerable amount of inert gas in the conductive elements did not slow down the rate.

A third tall vine was similarly given a 10-ml air embolus, followed by two hours of water. Again the rate remained unchanged. This vine was cut off 8 m above ground. Thirty ml ran out at the base, flushing out ¼ ml of air. Piece by piece the vine was cut down until practically all water had been drained down into the burette at the base. The total yield of water was 350 ml and with it came only 2 ml of air. It was clear, therefore, that at least 9¾ ml of air ascended up the vine through the valves. How and where it lodged is not known.

Tall Vines Wilted by Air: In two more experiments vines of 12 m and 17 m were used (fig 13). On a hot sunny day when the stem pressure was only a small fraction of an atmosphere, the vines were cut off with one slash of the machete. The cut end was dressed in air, and after a few minutes was provided with a water bottle. Here there was a sizable air embolus introduced both by the elastic volume-rebound of the vine when the low internal pressure was relieved by the cutting in air and by the recession of the transpiration columns. The water consumption rate was followed, and on the next day, which was somewhat cooler, both vines were given 30 min of air. After only 15 min the first signs of wilting appeared, for the tendrils and leaves began to droop. After 20 min, wilting was quite apparent, and was pronounced after 30 min. Even this did not stop water consumption when the vines were again given water. The rate was reduced to one tenth, but within 20 min to an hour the leaves and tendrils became turgid again, standing out horizontally, as is normal. The transpiration was followed for 4 more days. It stayed on a reduced level and showed, as would be expected, a pronounced diurnal rhythm. This vine undoubtedly had a large number of its vessels blocked by air, so that much of the sap flow must have taken place through the finer structures between the vessels.

Water Uptake against Reduced Pressure: One- and two-year shoots were cut under water from the top of a tall grapevine, were attached to water-filled open potometers in the laboratory, and the water consumption was followed. The potometers were then filled completely with water and stoppered without trapping any air. Gas bubbles started to form immediately at the cut stumps. They rose and collected in the upper end of the tubes as the water level sank (fig 15 B). The water consumption proceeded at an undiminished rate (fig 14). At the end of the experiment the stems were cut letting water rise in the tubes. A small air bubble remained at the top of each tube from which final pressures of 0.05 and 0.06 atm respectively could be calculated.

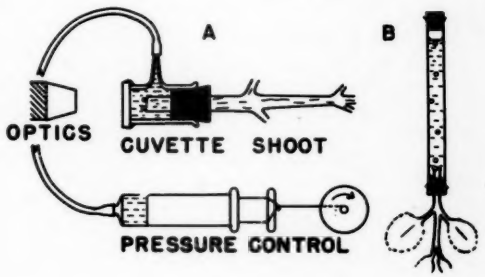


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FIG. 15. A. Pressure controlled cuvette to demonstrate cavitation of water in the vessels of a shoot. B. Water uptake in a closed potometer at reduced pressure.

In these experiments gas bubbles were liberated from the cut end of the stem. In order to see whence they came, a cuvette arrangement with pressure control was made (fig 15 A). By manipulating the pressure one could now easily demonstrate that most of the bubbles traveled out of the vessels. A great many vessels supplied bubbles, but whether all did, we do not know. Some shoots produced more bubbles than others. In these experiments, where the vines were forced to absorb water against reduced pressure, the water consumption did not diminish, in spite of the fact that a great many vessels were blocked by bubbles. In some vessels the bubble-trains even reversed the water flow.

Copper Sulfate Experiments: Strasburger (42, 43) demonstrated in a series of 21 experiments on seven species of trees and two species of vines (Wistaria and Hedera), ranging from 11 to 22 m in height, that solutions of copper sulfate, picric acid, carbolic acid, and

other strong poisons traveled up the trees to the very top in a few days. Big trees would consume up to 150 liters of the poisonous fluids. Water was always carefully flushed on the saw when the tree was cut to prevent breaking the water columns by air. We have modified his heroic experiments by breaking the columns first with an air embolus, and then giving them copper sulfate.

A vine 13 m tall, with the lowest leaves at 12 m, was given 20 min of air, until wilting started, and then a 10 % solution of copper sulfate applied. In a few hours a brown discoloration spread out from the veins of the hanging leaves. The vine was pulled down and cut up in meter-long sections. The blue fluid flowed freely in the vessels of branches 11 m up, and analysis showed the leaves to contain large amounts of the salt. Here neither poison nor a vascular transpiration stream broken by large amounts of air could prevent the vine from getting the fluid above atmospheric height and into the vessels and leaves.

In a second experiment a tall vine was connected with a burette and the rate of water consumption was measured every two minutes. After 20 minutes the water was replaced with 10 % copper sulfate. The consumption proceeded without any significant break in the curve, suggesting that there were no metabolic sap pumps at work in the stem.

Stability of Water Columns in Glass Tubes and Xylem Vessels when under Acceleration Pull or Vacuum: The method used below is a modification of that of Briggs (8a). Glass capillaries (0.2 to 1.3 mm bore), sealed at one end by fusion, were filled with tap water by spinning them under water in a centrifuge. They were then spun inverted, with the open end beneath a shallow layer of water or lukewarm India ink in a weak gelatine solution. The water in the capillaries did not cavitate during an hour's run at 1700 × g, which force developed in the 10 cm columns a pull of 17 atm. A capillary of 0.5 mm bore was sealed by dipping one end into melted (40° C) gelatin solution. When this was almost dry the capillary was filled with water in the centrifuge at 1000 × g. It then stood repeated accelerations, developing a pull of 10 atm on the water, without cavitation.

Fresh pieces of vine stem (approximately 1 × 11 cm) were sealed at one end by placing them in a centrifuge tube containing a 1 cm layer of warm, melted gelatine. They were spun in this for 3 min at 1500 × g and left to dry for some hours. They had now a 1-cm deep cavitation resistant gelatine plug in every vessel. The stem was evacuated in water for an hour, then placed under water in a centrifuge tube and spun at 1500 × g for 5 minutes, all of this to get rid of gas bubbles. The stem was now placed with the open end under warm India-ink-gelatin solution and spun at the desired acceleration. If cavitation took place in a vessel the water would drain down and upon deceleration the ink solution would ascend in it. It was found that practically all vessels cavitated between 1 and 1.5 atmospheres' pull. Only very few could take −2 atm.

In another series, a clamp was used, which obliterated two groups of vessels on opposite sides of the vine and left the rest open. The clamped stems had been evacuated in water and were spun at 1500 × g. Again the great majority of the clamped vessels cavitated under the slightest tension.

Finally, a V-shaped branch was flushed through with water, and both ends were placed in ink at the bottom of a centrifuge tube. Here again the great majority of the vessels had cavitated at 3 atmospheres' pull.

As a method for testing cohesion in vessels the above procedure is limited by the fact that the curvatures of all menisci flatten out and orient with increasing acceleration, which is probably detrimental to any valve action depending upon the menisci. Furthermore, it seems impossible to remove all gas nuclei even by prolonged evacuation and centrifugation, for after all this preparation, simple vacuum will still produce mass cavitation. On the other hand, what more potent means could the plant have to get rid of gas nuclei?

These experiments agree with the observations that many vessels of leafy grapevine shoots cavitated when the plants were forced to take up water against a reduced but positive pressure. We have accordingly good indications that tensile sap columns, if any exist in the vessels of the grapevine, are few and feeble.

Different conditions may obtain in the interstices, however. Dye experiments have indicated that part of the transpiration stream runs in these. A section of stem was found to contain 70 % water. Of this 25 % was in the vessels and ran out upon shaking. None of the remainder was dislodged by spinning the stem at 1700 × g. The fact that part of this water could be squeezed out shows that it was mobile and that it was locked by the interfaces at the cut ends.

Gas Content of the Sap: The experiment with gas emboli raises the question of whether or not a low tension gas phase coexists with the sap in the vessels. If such were the case, one would expect that the gases dissolved in the sap would become partly extracted on their way up through the stem. This has been tested by comparing the gaseous nitrogen contents of sap taken at various levels and times. Most of the samples were obtained by cutting out a meter-long section of the stem or branch of the intact vine with two rapid slashes. The lower end was put in paraffin oil under which the sap collected. Sufficient sap could easily and quickly be obtained by this method, and more could always be collected by trimming off an extra 10 cm at the top of the section. The sap flowing out under the oil was never accompanied by gas bubbles, even when the twigs were taken high up, during vigorous transpiration. At night samples could be drawn with a syringe attached to the manometer punch, as already described. The gas content of the sap was analyzed by a gasometric method requiring 1 ml samples (39).

TABLE I

GASES DISSOLVED IN GRAPEVINE SAP
1 ML WATER SATURATED WITH AIR CONTAINS 13.4 TO 12.2 MM³ N_2 AT 15 TO 20° C AND 6.9 TO 6.4 MM³ O_2

VESSEL PRESSURE AT GROUND LEVEL	HT OF SAMPLING	O_2	N_2
atm	*m*	*mm³/ml sap*	*mm³/ml sap*
0.07	0.5	5.1	13.4
0.8 *	0.1	4.2	12.7
0.2	9.0	4.0	12.0
0.2	7.0	4.2	11.9
0.12	8.0	4.4	12.5

* Night sample drawn by syringe.

From table I it is apparent that the nitrogen gas dissolved in the sap was always close to that of air-equilibrated water and that no significant extraction took place during the transport up through the stem.[4] We may therefore conclude that the water-conducting elements are completely sap-filled and are indeed well shielded from diffusion contact with any evacuated vessels on the long way up the stem. This is all the more remarkable inasmuch as mobile water permeates all structures between the vessels. These experiments also showed that the vessels in the small branches high up and during vigorous transpiration contain an abundance of sap.

POTOMETER EXPERIMENTS ON AIR-FILLED TWIGS: Several of our findings in tall grapevines are at variance with the idea of a simple cohesive system of vascular sap columns. They are, therefore, seemingly in conflict with the ingenious potometer studies of Renner, Nordhausen and others, where tension gradients as high as – 5 to – 10 or – 20 atm were often calculated. A potometer gives the rate of water uptake which the plant produces across a resistance as compared to the rate a vacuum pump produces through the same resistance. Assuming proportionality between rate and pressure difference, the latter can be calculated. So far the conclusions seem safe. The highest pressure gradients were obtained with plants more or less deficient in water. On the plant side of the resistance there was, therefore, dehydrated tissue which might well develop strong imbibitional or even osmotic forces, whether the vessels were functioning or not.

This possibility was tested on 2-year shoots of greenhouse camellias (*Camellia japonica*), obtained through the courtesy of H. V. Lawrence, Inc., Falmouth, Massachusetts, and on leafless stems of *Clematis virginiana* L. and *Smilax rotundifolia* L. taken in the woods in January. In all of these experiments tension in the vessels was eliminated by filling them with air.

The single-clamp method of Renner (35) was employed, using stumps only 1 cm long to minimize evaporation and residual suction. The relative humidity was 40 to 43 %, temperature 24 to 26° C. The vacuum pump, in series with a moist chamber, delivered a suction of 72 cm Hg.

Camellia A: A shoot with 20 leaves was connected end to end with a capillary of 300 mm³ capacity containing a water index drop. The shoot took in 150 mm³ air in 2 hours and unmeasured air for 4 more hours. The plant developed a gradient of 5.6 atm across the clamp resistance (fig 16 A).

Camellia B: A shoot with 10 leaves took in 270 mm³ air overnight. It produced a gradient of 15 atm through the clamp resistance (fig 16 B).

Camellia C: On a shoot with 15 leaves the stump was plugged with grease and left in air overnight. The leafy top and the base were cut in air, leaving 10 leaves. The stem was therefore open at both ends and contained some air. Nevertheless, the stem produced a drop of 20 atm across the resistance (fig 16 C).

Clematis Stem A: Bare stems, moist and fresh, ½ m long were taken into the laboratory from the woods. Both ends were cut. The vessels were filled with gas and could be blown through. The gradient was 4.3 atm across the clamp resistance (fig 17 A).

Clematis Stem B: The conditions were the same as for the foregoing. The gradient was 2 atm (fig 17 B).

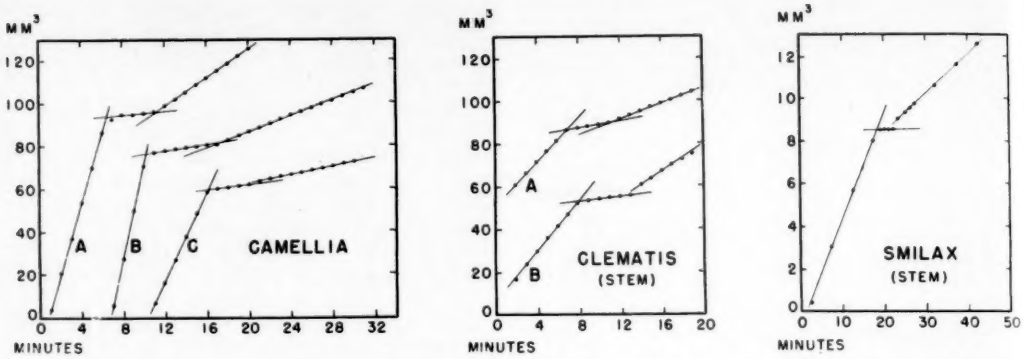
Smilax Stem: This was brought in from the woods, with no leaves, but fresh and moist. The vessels were filled with gas. Both ends were cut. The gradient was 2.8 atm (fig 18).

These experiments show that high tension forces can be produced in twigs even though the vessels are open and filled with air.

In the cases where twigs of intact trees were clamped during a period of assumed tension (27, 38), it seems inevitable that crushing the twig by the clamps would have broken the columns, for great delicacy in manipulation has otherwise proved to be imperative to avoid such breaks (6, 10). One may therefore say that, although potometer experiments have proved that tension gradients of several atmospheres may be produced experimentally in twigs of tall trees, they have not proved that this pertained to the vessels.

Nordhausen (30) conducted a series of potometer experiments on trees. He used an ingenious ceramic resistor between the branch and the potometer and compared (a) the amount of water the branch could draw through the resistor with (b) the amount a pump could draw through the resistor. The amount of water disappearing from the potometer in (a) was only 0.5 to 1 mm³ per min. The system was sealed to the branch with wet clay covered with tinfoil. The potometer water was therefore in direct communication with the water of the wet clay body and bark. It would seem impossible to decide by what route the water disappeared. Also in the twig experiments it would seem difficult to evaluate the relative role played by cohesion in vessels as compared to imbibition and evaporation from the twig.

[4] The oxygen content of the sap was strongly influenced by the length of time between sampling and analysis. Oxygen disappeared in stored sap samples and thus the values in table I are minimal ones.



FIGS. 16 to 18. Potometer experiments. The first slope of each curve is the rate at which the clamped plant takes water from the potometer, the second slope is the rate of water absorption of the stump, and the third slope is the rate produced by attaching a vacuum pump to the stump.

Fig. 16 (*left*). Camellia shoots, A and B containing air in the stem, C with stem open and containing air.

Fig. 17 (*center*). Clematis stems, both (A and B) having vessels filled with air and open.

Fig. 18 (*right*). Smilax stems, vessels filled with air and open.

DISCUSSION

When the vine is analyzed as a static system, i.e., when there is no sap transport in the stem (in the springtime, at night, or in isolated sections), we have found the following. The vessels in a fresh vine contain a great deal of sap throughout the height of the vine. The sap from sections of any part of the vine runs out of the vessels unaccompanied by gas bubbles and is fully saturated with atmospheric nitrogen. It is, therefore, contained in the vessels and is well shielded against direct or indirect diffusion contact with a low pressure gas phase, if such exists. In the springtime and at night, the sap in an intact vine exhibits a normal hydrostatic gradient of positive pressures, and if these are not too low sap can be withdrawn from the stem. A detached stem filled with water also presents a normal hydrostatic gradient, and the water in it flows easily. The great majority of the vessels have valves spaced approximately 60 cm apart. These will pass water freely, but not a water-air interface. A certain number of vessels lack functional valves over at least several meters.

During transpiration the picture changes. The manometer punch indicated the absence of a hydrostatic gradient in the vessels and never showed pressures as low as the water vapor tension. It was not possible to draw sap samples out of a transpiring vine. The rate of sap transport was not affected by copper sulfate. The introduction of considerable amounts of air or inert gas into the vessels of vines taller than 10 m, severing all vascular columns active in the transport, did not even slow down the water consumption rate. Leafy shoots taken high up on the vine consumed water at an undiminished rate when the water was put under vacuum, but trains of gas bubbles would then emerge from a great many vessels. Likewise the water in the vessels of a stem cavitated when put under the slightest acceleration tension. When the vessels of a vine stem were plugged with mercury for some 50 to 60 cm, the interstices between the vessels would still conduct water, but at a considerably reduced rate. We have also shown by potometer experiments and acceleration tests that high tensions can develop in the interstices, but there is no proof as yet that these exist in the vessels.

Amongst the observations above several harmonize with the cohesion theory: the evidence for continuous, freely mobile sap columns, the absence of metabolic pumps, the fact that sap could not be extracted from the stem during transpiration and that the water consumption rate in shoots was independent of pressure.

Other data are not easily interpreted in terms of the cohesion theory. The manometer data seem eminently valid at night, when it cannot be accidental that they record precisely a hydrostatic gradient. They would give a distorted picture of the sap pressure in the daytime if a large number of vessels filled up with low pressure gas during transpiration. This is a possibility, but one may then ask why the sap does not lose dissolved nitrogen on its way up the stem. Considering that a large amount of sap runs in the vessels during transpiration, it is also striking that we never recorded pressures as low as vapor tension, which would be suggestive of breaking into banks of cohesion columns. Furthermore, there is no mechanism in sight by which a steady supply of extracted nitrogen could be eliminated daily in a vine over 10 m.

As to the gas emboli experiments one may object that, even though we know that practically all of the gas passed the lowest valves, we do not know what happened after that. It could conceivably lodge in a staggered fashion in the lower part of the vine, letting the sap pass by in the tissue between the vessels. Cohesion might possibly overcome this increase in resistance. If the air moved high up in the vine, forcing the valves behind the retracting sap columns, then a

transport system depending upon cohesion in the vessels would surely get jammed by the expanding gas.

In vitro observations indicate that water in stems and shoots cavitates at vacuum or slight tension. Here one may argue that perhaps some vessels did not cavitate. This may be so, but the presence of only a few strands of cohesive columns would hardly explain the relative abundance of sap which we find in the vessels of high branches during transpiration.

When a vine is wilted by cutting the stem, more and more air will be aspirated into the stem. Even with the leaves in an advanced state of wilting, one may still find sap in the vessels of some cut-out sections. Obviously these sap columns were isolated from the leaves by breaks in the columns. In dehydrated material like this, potometer experiments have shown that strong cohesive or osmotic forces are likely to develop, and it is difficult to see how tension in the interstices could exist without being transmitted to the sap in these vessels. Therefore, when we find sap-filled vessels in a wilted vine we might expect the sap to be under tension. We have been unable to demonstrate, however, that such a tension can exist, and we certainly do not know that it obtains in a normal transpiring vine. We may similarly argue that if the transpiration stream runs mainly in the interstices and is lifted by cohesion this would require a very high tension pull in the upper parts of the plant in order to overcome the resistance, but, if this is the case, how can the plant maintain an abundance of sap in the *vessels* high up?

It would be of great interest to investigate other wide-vesseled but much taller vines climbing 30 to 40 meters up into the region of supposedly perennial cohesion. However, even at the relatively modest levels of our grapevines the measurements and observations are, on several scores, difficult to reconcile with the idea of tensile sap strings in the vessels, and we would not venture to make any guesses as to what one might find in vines many times taller.

There is at the present time no knowledge that sap columns do exist or even could exist in the vessels of high trees at the postulated tensions of -20 or even -100 atm. There are indications to the contrary from the grapevines. There is no mechanism known which could repair accidental breaks in such columns high up, and there remain both old and new observations which are difficult to reconcile with the theory. In its application to tall trees and vines one may therefore describe the present situation by saying that the fascinating old problem is still essentially unsolved.

Summary

The hydraulic relations in tall vines of *Vitis labrusca* L. have been investigated. The pressures in the xylem vessels were measured directly, using a manometer punch. When filled with water the vine stem acts like a simple water hose, exhibiting a normal hydrostatic gradient. Most vessels in the stem have functional valves spaced 60 cm apart which will freely pass water, but not an air-water interface. In the spring before the leaves are out, the sap pressure is often 3 to 4 atmospheres and a normal hydrostatic gradient of 0.1 atmosphere per meter elevation obtains in the stem. During transpiration the pressure at ground level is often only a fraction of an atmosphere and a hydrostatic gradient is not found. Yet pressures were never as low as water vapor tension, which would have been indicative of cohesive columns. The sap at all levels is saturated with atmospheric nitrogen and hence probably travels up the vessels without diffusion contact with a low pressure gas phase. Sap could not be withdrawn from the stem at low positive pressures, but came out easily when the pressure approached atmospheric. Leafy shoots consumed water at an undiminished rate when vacuum was applied to the water, but at this pressure trains of cavitation bubbles emerged from many vessels. Vessels in the stem did not support experimentally produced tensions. A considerable volume of air or helium was aspirated by the cut stems of vines, severing every vascular water column carrying the transpiration stream. Yet this did not even temporarily slow down the rate of water consumption in a vine taller than ten meters. Most of the air travels up the vine, but how far is not known. A tall vine wilted by a large amount of aspirated air will resume water consumption at a much reduced rate and become turgescent again. Such a wilted vine will also draw copper sulfate up into the leaves. Potometer experiments showed that high tensions across a resistance are also readily obtained when the vessels are filled with air, and such experiments consequently do not prove cohesion in the xylem vessels. Several of the above and earlier findings are, at least for the present, not explainable in terms of the cohesion theory. Ultimate clarification of the old problem of sap rising in tall trees hinges upon the difficult task of obtaining reliable hydraulic pressure gradient measurements in interstices and vessels of trees or vines taller than ten meters.

We are indebted to Mr. Carleton Grant, instrument maker, at the Woods Hole Oceanographic Institution, for skillful craftsmanship in the construction of the manometer punch.

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THE RELATION OF EXTERNAL RUBIDIUM CONCENTRATION TO AMOUNTS AND RATES OF UPTAKE BY EXCISED POTATO TUBER TISSUE [1,2]

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A number of studies have indicated that the amount of salt taken up by plant tissue corresponds to the external concentration in the manner of a Freundlich adsorption isotherm (for references see 2, 10, 28). The idea that adsorption is involved in uptake seems to be gaining general acceptance at least for the initial phase of uptake (2, 16) despite the fact that the validity of the relation and of the concept—as a part of the accumulation mechanism—has been questioned (12, 28). The present report is concerned with the relation of ion concentration in the external solution to the amount of uptake by excised tissue, as possibly an adsorptive process, and the probable significance of this relation to accumulation.

The fact seems well established that the time course of cation uptake consists of at least two, easily distinguished, time phases: 1) an initial non-metabolic process which is presumed by many to be an adsorption, adsorption-exchange, or exchange process (1, 24, 26, 28, 30, 32), and 2) a metabolic process, which is dependent on aerobic respiration and in which accumulation may proceed against a concentration gradient (2, 3, 4, 17, 21, 28, 36). Phase one of the uptake, or loss, is essentially independent of temperature and aeration as shown by isotopic exchange of potassium (5); it corresponds in exchange to relative concentration of two ions as expected by the law of mass action (36); and it shows ion selectivity (13, 22). Phase two of the uptake has a high temperature quotient ($Q_{10} = 1.5$ to 3.0) (4, 33) and is dependent on oxidative metabolism; it has been reported to be directly dependent on external concentration (14, 20, 23, 34) though evidence to the contrary has been reported for intact plants (18).

In former years there was a strong tendency to view initial ion penetration as a diffusion and permeability process (3, 27); however, more recently a trend has developed to consider the first steps in uptake to be a binding reaction, e.g., an ion carrier complex, either in a non-metabolic process or in energetically coupled reactions (27). This view has received substantial support recently with the demonstration that in some cases of related ions the effect of one ion on another in uptake by roots fits the Michaelis-Menten equation for competitive inhibition as in an enzymatic process, whereas with other ions uptake is uncompetitive (8, 9). This concept seems not to be inconsistent, at present, with the hypothesis that an initial step in ion uptake may be best described as an adsorptive process.

Osterhout (19) has previously reported that NH_4^+ uptake by the alga, Valonia, is not in proportion to the concentration gradient as expected if it is primarily a diffusion process. He concluded that NH_4^+ probably entered cells in chemical combination with some substance, X, acting as a carrier; then rate of uptake was directly proportional to NH_4X concentration. Stiles and Kidd (31) reported that salt uptake in long-term experiments (2 or more days) by potato tuber discs responded to external concentration in amounts approximating a fit to the Freundlich adsorption equation. Stiles later (30) refers to this equation and the fit of salt absorption by tissue to it as approximations applicable only to the second phase (in time) of uptake. Several other reports in which uptake conforms to, or approximates, an adsorptive process include: the uptake of bromide ions by Nitella (12); the uptake of bromide ions by discs of potato tuber (28); the uptake of phosphate ion by intact plants (35); the uptake of rubidium and bromide ions by potato tuber tissue (28); the uptake of phosphate ions by intact maize plants (10).

The criticisms against the concept that an adsorptive process may play an important role in ion accumulation have been: 1) that uptake does not closely fit the expected curve (28, 30); 2) that the concept implies that adsorption itself is the concentrating power, whereas protoplasmic activity seems required to supply the driving force (11, 12, 28); and 3) that the adsorption equation (Freundlich) is itself only an approximation (30) or too general (12) to be of aid in any explanation of ion accumulation. With regard to criticism 2 above, there appears to be no real conflict between the idea that adsorption may play an important role in ion accumulation and the fact that accumulation requires energy from oxidative metabolism. In fact, the chief attraction of the concept of an adsorptive process is the fact that it supplies some explanation of the selectivity of protoplasm in its absorption processes (4). Criticism 3 above seems largely dependent upon criticism 1 because if uptake does indeed conform to any equation, any mechanism postulated for the absorption process must account for the relation, whether it is in terms of adsorption or a quantitatively similar process. At present, then, there appears to be no reason why adsorption should not be involved in uptake. The present study was made with rubidium and potato tuber tissue to pro-

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vide additional measurements of the quantitative nature of uptake as related to external concentration. These measurements should serve as a test of the goodness of fit to an adsorption equation but do not, of course, provide critical evidence to distinguish adsorptive from other processes which might fit a similar equation.

Materials and Methods

The experimental plant tissue consisted of excised discs of potato tuber tissue, *Solanum tuberosum* L., varieties Katahdin, or California Long Whites. Discs were cut in a Bausch and Lomb hand microtome using a cylinder of tissue extracted from the central portion of a tuber by a cork borer. The disc size is indicated for each experiment. The discs of tissue were thoroughly washed in tap water or distilled water and—with the exceptions noted—were submerged in vigorously aerated distilled water 24 hours preceding each experiment.

During the tests, the discs were submerged in solutions of rubidium chloride labelled with radioactive Rb^{86}. The usual containers were 250-ml Erlenmeyer flasks fitted with rubber stoppers and glass aerating inlet and outlet tubes. Aeration was at a rate of 3 l/min. Ordinarily 10 or more discs were placed in 100 ml of the experimental solution giving about 6 gm or less per 100 ml of solution.

In respiration tests, manometric measurements were made with Warburg vessels having a center well and a capacity of about 15 ml. In these tests 12 discs, 1×7 mm, were used in 1.5 to 2.0 ml of solution. The flasks were shaken at a rate of 125 oscillations/min in a path of 4.5 cm.

The experiments on uptake and respiration were conducted at 25° C.

The use of Rb, which resembles K in absorption, as a measure of cation uptake seems justified by previous work (6, 9, 28). Uptake of Rb was measured by the use of radioactive tracer Rb^{86} obtained from Oak Ridge National Laboratory in 10 mc units as irradiated Rb_2CO_3.

All samples for counting were dried in #0 Coors porcelain capsules. Tissue samples having a dry weight up to about 200 mg were also placed in these capsules for wet ashing with concentrated nitric acid at a moderate temperature. After heating gently to dryness once or twice, some hydrochloric acid was added to the nitric acid. This process was found to be rapid and accurate even without the complete oxidation of carbon. Tests of recovery of added Rb^{86} revealed an accuracy within $\pm 2\%$.

Experimental Results

Three tests on the effect in time of external concentration of Rb^+ uptake were run. In each case samples were taken at several intervals over a period up to 48 to 96 hours. Conditions of the three experiments are given in table I.

In experiment 1, the tissue samples were sufficiently small that relatively little change in external concentration occurred except in the weakest solution; the thinner discs of this test would be expected to show the greatest effect of internal concentration on the Rb^+ absorption. In experiment 2, the design was such that both uptake by tissue and depletion in the external solution could be followed. Experiment 3, in which a small volume of solution was used and Rb^+ depletion measured, constituted both a measure of an extreme effect on change of external solution, and a measure of uptake by discs.

The Time Course of Absorption at Different Concentrations: Previous work has shown that potato discs transferred to a solution of RbBr show an initial time phase of very rapid uptake of cation followed by a second phase in which uptake of both ions is slower but at a relatively constant rate over many hours (28). The time course of Rb^+ uptake in the present tests confirms this finding, with respect to the cation, at a wide range of concentrations except that, in time, absorption decreased in rate. The results of experiment 2, which seem typical, are shown in figure 1. The first phase is characterized by its initial steepness and gradual change of slope; in the experiment illustrated, the first phase appeared to end within 2 hours at the lower external concentration whereas at the higher concentration it lasted over 7 hours. In both phases, it appears that uptake was a function of the concentration.

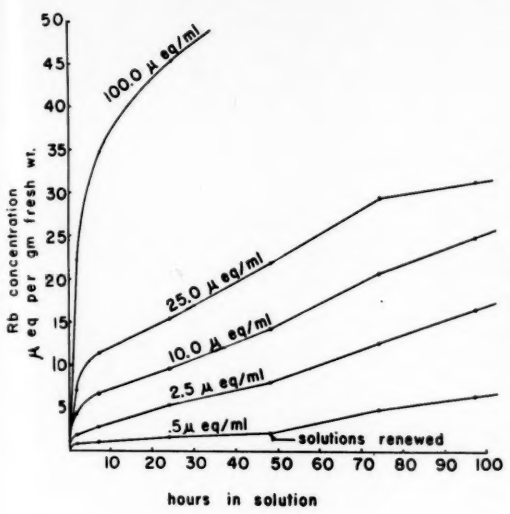
It is a striking feature that the inception of the second phase in the time course of uptake appears to bear no direct relation to the concentration gradient. Thus phase two may be seen in figure 1 to have started in 7 hours at the higher concentrations under

Table I

Conditions in Three Tests of the Effect of Concentration on Rb Uptake by Potato Tuber Discs

Exp't	Disc size		No of discs in sample	Replicates	Sol'n sampled	Pretreatment, hrs washed	Hour intervals	Conc range ext sol'n μEQ/ml	Gms tissue fresh wt	Vol sol'n, ml
	Diam	Thickness								
1	20	0.75	1	2	No	48	0.5, 1, 5, 24, 48	0.007–25.0	2.5	100
2	15	2.0	2	5	Yes	96	2, 7, 24, 48, 74, 97	0.50–100.0	28.0	200
3	7	1.0	(60) *	2	Yes	48	0.17, 4, 26, 48.5, 68	0.056–25.0	2.75	20

* Rb^+ absorption was followed by assay of aliquots from the external solution only; tissue concentration was estimated by difference.



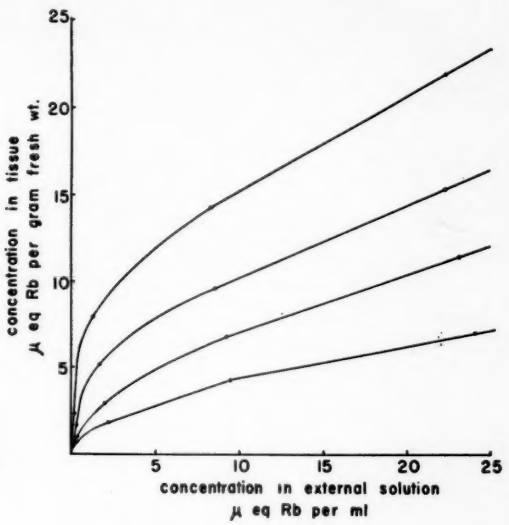


FIG. 1 (*left*). The time course of Rb uptake by potato tuber discs (exp't 2) at various concentrations of RbCl in the external solution.

FIG. 2 (*right*). The concentration of Rb in the tissue as related to external concentration at each of several intervals of sampling. Data from exp't 2.

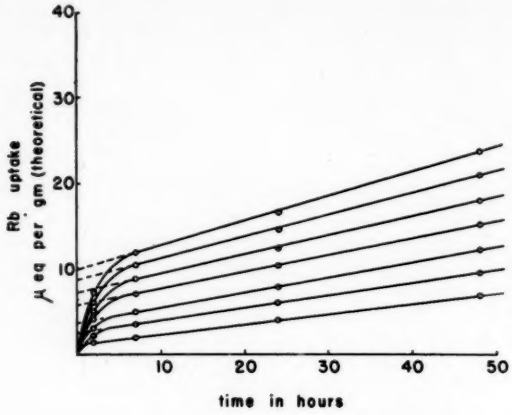
conditions in which the external Rb^+ concentration greatly exceeds that of the tissue; whereas at the lower concentrations the accumulation was proceeding against a concentration gradient in 7 hours. Therefore, it may be inferred that the accumulation mechanism controlling Rb^+ absorption operates at essentially the same pace both with or against a gradient; and, it appears to be a function continuous with and closely related to the height of phase one. Thus it seems to be indirectly related to the external concentration.

The sensitivity of Rb^+ absorption rate to external concentration is well illustrated by the response to the solution renewal at 48 hours (fig 1). An increase in rate of uptake occurred with the restoration of the external solutions to the initial values; although it is not revealed in the curves of figure 1, the increased uptake following solution renewal followed the course expected if phase one uptake recurred. This response is significant, however, because it occurred at various accumulation levels, in spite of the direction of the concentration gradient.

THE APPROXIMATION OF PHASE ONE RB UPTAKE VALUES AS RELATED TO EXTERNAL CONCENTRATIONS: The time course curves shown in figure 1 are not strictly accurate since they are plotted against the initial (uncorrected) value of the external concentration. In time, as the discs absorbed Rb^+, the external concentration decreased. A close approximation of true values may be obtained graphically by plotting tissue concentration directly against external concentration as experimentally measured at each of the sampling intervals. These curves are shown in figure 2. From figure 2 may be seen the relation of the amount of Rb^+ in the tissue at any given time to the external concentration at that time. The coordinates on the curves of figure 2 may then be plotted as in figure 3 for any concentration, within the limits measured, at any of the sampling intervals used. Figure 3, then, should closely approximate the amount of Rb^+ taken up by the tissue at a given constant external concentration. It should, therefore, more accurately reflect the true course of Rb^+ absorption from concentrations at different levels. From this curve, as seen below, further approximations may be made with regard to the amounts of Rb^+ taken up in the initial phase.

If adsorption, or adsorption exchange, is a normal component of the uptake mechanism, it might be expected in particular to occur during the initial phase of uptake. The maximum amounts of phase one uptake cannot be measured directly, of course, since no true equilibrium is attained—rather, in the time course, phase one uptake passes gradually into phase two. However, a close approximation can be made of the maximum Rb^+ uptake values of phase one at any external concentration by extrapolation of the phase two uptake curves back to zero time (fig 3). This is based on the assumption that phase two uptake is linear in time and that it proceeds from zero time essentially at the same rate.

A plot of the derived phase one "equilibrium" values is shown in figure 4 in which the $\log_{10}$ of internal concentration is plotted against the $\log_{10}$ of external concentration. This is the form of the Freundlich adsorption isotherm, $y = kc^n$, or $\log y = \log k + n \cdot \log c$, where y is the amount absorbed, c is the external concentration and k and n are constants.



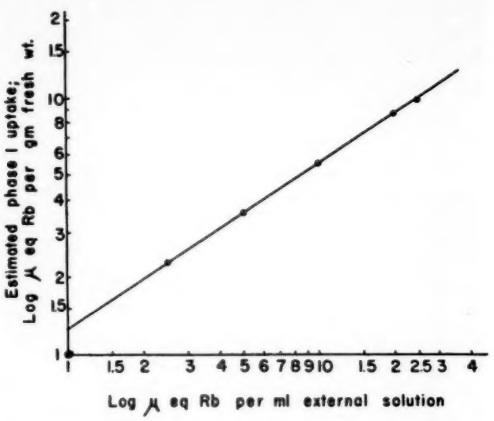
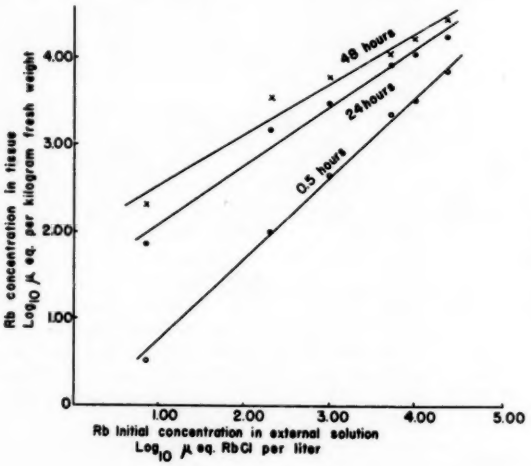


FIG. 3 (*left*). Family of hypothetical time course curves for concentrations 1.0, 2.5, 5, 10, 15, 20, 25 μeq Rb/ml. The points were derived from the corresponding coordinates of figure 2 (data from exp't 2). They approximate the amount of uptake expected at any moment at constant conc. The extrapolations back to zero time approximate the "equilibrium amount" of the initial uptake process—possibly adsorptive—of phase 1. See text.

FIG. 4 (*right*). A plot of the $\log_{10}$ values of Rb uptake by tissue versus the $\log_{10}$ values of external conc. The points are theoretical having been derived from the zero time intercepts of figure 3 (data from exp't 2). The curve corresponds to the Freundlich adsorption isotherm.

The fit of the points is excellent except at the lowest concentration (fig 4). It, therefore, appears that the initial phase of uptake corresponds well over a range of concentrations to the equation describing an adsorption process of the Freundlich type.

RELATION OF TOTAL ABSORPTION TO EXTERNAL CONCENTRATION: The log vs log plots of various intervals of experiments 1 and 2 are shown in figures 5 and 6. The goodness of fit seems to be close in the data of all three experiments and at any given time of sampling. This was over a concentration range of 0.007 to 25.00 meq/l in experiment 1 and of 0.37 to 100.0 meq/l in experiment 2. The fit to the log vs log adsorption line is better if the 100 meq/l treatment is omitted; this is not an argument against the correlation, however, because the discs in this solution did not behave normally. (They lost some of the brown color typical of healthy potato discs, and at the end of 48 hours when they were discontinued were observed to have decreased appreciably in fresh weight.


Rb concentration in tissue
Log10 μ eq. per kilogram fresh weight
48 hours
24 hours
0.5 hours
Rb initial concentration in external solution
Log10 μ eq. RbCl per liter

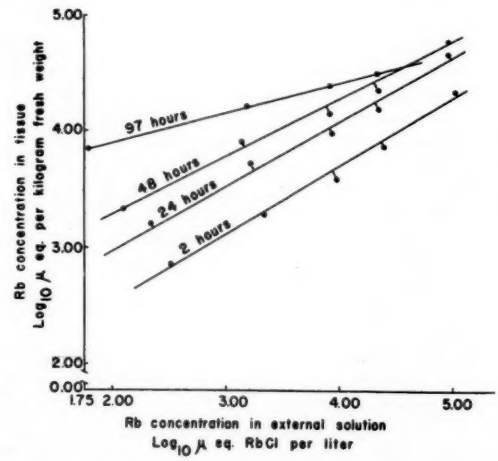


FIG. 5 (*left*). The log vs log plots of internal concentration against external concentration as a test of fit to the Freundlich adsorption isotherm. Data of exp't 1.

FIG. 6 (*right*). As figure 5 but data of exp't 2.

This is not unexpected at higher concentrations of a single salt solution). The greater uptake, or influx, of Rb^+ at the 100 meq level, as shown on the log vs log plot, is attributed to a partial failure of the protoplasm to limit the entrance of Rb^+ in a normal manner.

The results of regression analyses as a statistical test of fit of Rb^+ uptake to the Freundlich equation are shown in table II. The coefficients of correlation are high and indicate that this equation at any given time quite closely describes the relation of Rb concentration in the tissue to that in the external solution.

RATES OF UPTAKE: After phase one, the rates of absorption at any given concentration were nearly constant over many hours (fig 1, 3, 7). However, the constancy of rate as shown in figure 3 derived curves) is somewhat illusory since the change in slope of the plots showing tissue concentration (log vs log plots, fig 5 and 6) clearly indicate that in time there is a decrease in response to external concentrations.

The effect of solution renewal in experiment 2 at 48 hours clearly greatly increased the rates of uptake and instituted a second phase of rapid uptake (fig 1). In the following period 74 to 97 hours), however, absorption rates compared closely with those of the 24- to 48-hour period (fig 7). An exception was the highest concentration in which the rate dropped considerably. Apparently, this was due to the approach to maximum uptake as found with K^+ absorption by beet tissue (32).

In the later part of the experimental period, external concentration appeared to have little or no effect on absorption rate except at the lowest concentration level (fig 7) in which accumulation in the tissue never reached a high (absolute) level. The data suggest, therefore, that potato tuber tissue has a maximum capacity for cation retention; upon approaching this level, then, concentration of the external solution over a wide range may have no effect on absorption. Indeed, such a phenomenon has been reported over certain concentration ranges by Olsen (18) for absorption of anions by intact plants and by Overstreet et al (20) for K^+ uptake by excised roots. Olsen found that in conditions in which the proportion of different ions were constant, the amount of a given ion absorbed per unit time over a wide range of concentration (e.g., for NO_3, from 14.0 to 0.0003 meq/l). From the present data on potato tuber tissue, it seems likely that a similar constancy of uptake not affected by external concentration might be found under conditions in which the tissue has nearly attained a maximum capacity. It is apparent that after the first 2 hours—despite some differences in absolute uptake in conformance with the concentration gradient—the rates of uptake rather closely approached a constant value (fig 7). This seems to be an indication that Rb absorption after the initial phase is quite uniform in rate over a wide range of external concentration and despite the direction of the gradient. This would be expected if metabolically driven absorption is a function of a surface area which is being turned over at a constant rate and on which ions are adsorbed in accordance with the external concentration.

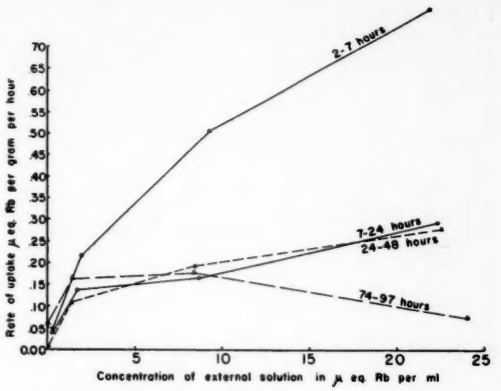


FIG. 7. The rates of Rb uptake by potato tuber discs at different intervals of time as influenced by external concentration. Data of exp't 2.

The relation of phase two uptake rates to the amounts of Rb^+ taken up initially is shown in figure 8. Both of these values are, in a sense, hypothetical, being derived graphically from figure 3. However, it appears that the amount of Rb^+ taken up by discs in the first phase in time directly governs the amount which can be taken up per unit of time. This might be expected if accumulation is mediated by an adsorber from which the ion must be accepted by the active absorption mechanism.

LOSS AND EXCHANGE OF ABSORBED RB: In the previous experiments Rb^+ uptake was measured under conditions in which a new cation was presented to the tissue. Since potato tissue initially has no Rb^+ (or only trace quantities), the initial uptake rates and permeability constants for inward movement could

TABLE II

REGRESSION ANALYSIS OF THE DATA SHOWN IN FIGURES 5 AND 6

HRS	a	b	REGRESSION COEF, r	STANDARD ERROR OF b (= s_b)	t $\left(=\frac{b}{s_b}\right)$	DEGREES OF FREEDOM
			Exp't 1			
0.5	−0.1832	.9289	.998	.030	30.98	4
5.0	+1.0221	.677	.974	.079	8.569	4
24.0	+1.4315	.663	.990	.029	22.86	4
48.0	+1.9795	.573	.984	.053	10.83	4
			Exp't 2			
2	+0.4939	.5954	.996	.033	18.04	3
24	+1.0267	.5260	.992	.038	13.77	3
48	+1.3061	.4705	.993	.032	14.66	3
97	+3.3329	.270	.997	.014	19.28	2

In the formula for the regression line, $y = a + bx$, y and x are the $\log_{10}$ values of internal and external concentrations respectively; a and b are constants for any given line.

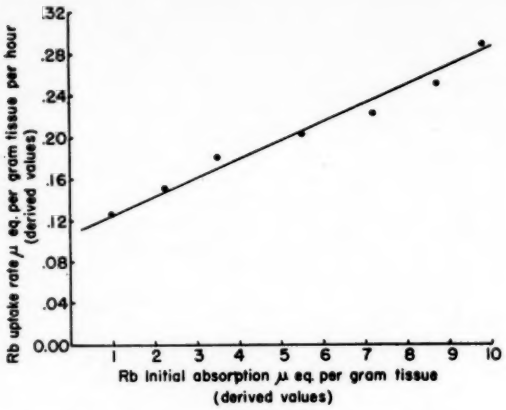


FIG. 8. The hypothetically proportional relation of rate of Rb uptake to the amounts of initial adsorption. Both values are derived: rates from the slopes of figure 3, and adsorption values from the intercepts at zero time.

differ markedly from those measured by loss or isotopic exchange of tissue having previously absorbed Rb^+. Consequently, experiments were performed to ascertain the magnitude and rate of loss of absorbed Rb^{86} from tissue transferred to conductivity water and to stable $Rb^{85}Cl$ solution.

In these experiments, potato discs, 1 mm thick and 19 mm diameter, were placed in labelled RbCl solutions, 5 meq/l for 24 hours. They were then rinsed quickly (about 10 sec) in conductivity water, blotted and placed in conductivity water or stable RbCl solution for various intervals of time.

The experiment on loss in water was separate from that involving exchange. However, the time course of percentage loss of Rb^{86} from the tissue is shown for both experiments in table III. In these tests, the amount of Rb^+ loss in percent was about the same during the first 2 hours whether due to net loss to water or to exchange for stable Rb. In the 24-hour period, there was no subsequent loss to water; however, isotopic exchange continued and reached a total of 41 % loss.

The results of these tests indicate that at any time an appreciable quantity of Rb^+ was loosely held by discs and was readily lost. The remainder of absorbed

TABLE III

LOSS OF Rb^{86} FROM TISSUE TO CONDUCTIVITY WATER OR BY EXCHANGE FOR STABLE RB

TIME, HRS	LOSS IN WATER		LOSS IN RbCl SOLUTION	
	μEQ/GM DRY WT (± STD ER)	%	μEQ/GM DRY WT (± STD ER)	%
0.0	55.4 ± 1.57	0.0	41.8 ± 1.12	0.0
0.5	43.5 ± 0.44	21.5	34.1 ± 0.62	18.4
2.0	42.4 ± 1.67	23.4	32.9 ± 0.72	21.3
24.0	43.0 ± 1.84	22.4	24.7 ± 0.79	41.0

Rb^+, however, was more firmly held and was exchanged for stable Rb^+ but was not readily lost to distilled water. The rate of exchange after the initial phase was not rapid but rather proceeded at a pace to be expected of a low outward permeability. Calculating the permeability constant, P (see 3), for the outward movement of Rb:

$$P = \frac{\text{moles} \times \text{cm}^{-2} \times \text{g}^{-1} \times \text{s}^{-1}}{\text{moles conc gradient}}$$

during the interval 0.5 to 24 hrs: P efflux = 2.38×10^{-11} moles cm^{-2} g^{-1} s^{-1}/M; and during the 24-hour uptake period: P influx = 1.56×10^{-9}. These values are of the order of magnitude expected in an accumulation process. The relatively small value for P efflux, as compared to P influx, in exchange of Rb^{86} for stable Rb^{85} ions indicates that the process is paced by a transfer mechanism.

RESPIRATION AND RUBIDIUM UPTAKE: Since salts are known to induce increases in respiration, tests were made to see whether or not different RbCl concentrations influenced the "adsorption" relation previously found due to some effect on respiration. No stimulation of respiration was found in eight hours over the range of 0 to 40 meq RbCl/l as shown in table IV. In fact, the respiration of the controls in water exceeded that of any RbCl concentrations. It, therefore, appears that, if anything, Rb^+ may slightly depress oxygen consumption, although none of the differences noted are considered to be significant. In any case, it is believed that RbCl had no significant influence on respiration in the experiments reported here.

TABLE IV

RESPIRATION RATES AND AMOUNTS OF RB UPTAKE BY POTATO TUBER DISCS IN DIFFERENT CONCENTRATIONS OF RbCl. DISCS PRETREATED 24 HRS IN DISTILLED WATER

TREATMENT RbCl μEQ/L	RESPIRATION		RB UPTAKE	
	μL O_2 PER GM PER HR (8 HRS)	% OF CONTROL	μEQ/GM FRESH WT	μEQ Rb / μEQ O_2
0.0 (control	126	100	0.00	0.00
1.0	124	99	1.03	0.10
10.0	111	88	9.60	1.14
20.0	119	95	19.0	1.92
30.0	121	96	27.0	2.67
40.0	123	98	34.1	3.32

The rates and amounts of Rb uptake corresponded well with those of previous tests and give a straight line with the log vs log plot of internal against external concentration.

DISCUSSION

The present data give further evidence which is consistent with the hypothesis that adsorption may be an important mechanism in cation uptake by tissues. The relation found between the amount of uptake and the external concentration of Rb quite

closely corresponded to the Freundlich adsorption equation $y = kc^n$. This relation held during the initial uptake phase when external concentration exceeded internal concentration and also during the later periods when accumulation proceeded against a concentration gradient.

Arisz (2) expresses the view that an adsorption equilibrium exists in the protoplasm and that this may limit the amount of ions secreted into the vacuole. The present results are in accord with this view. Thus amounts of Rb^+ accumulated by the tissue seems to be a complex function of external concentration but absorption rates approach a simple function of the amounts taken up in the initial uptake phase; this might be expected if initial cation uptake is adsorptive in nature and seems to be in accord with an idea of Osterhout's (19).

Calculations of permeability constants of potato tuber tissue for Rb^+ have shown that permeability is less in stronger RbCl solutions; similar results have also been reported by Krogh (15). This is in accord with the idea that the number of sites or binding units in active transport is limited to any given respiration level; thus permeability inward may be high in low external concentrations with a high binding site number to Rb^+ ratio but decrease markedly at lower ratios. Such a mechanism might be expected under appropriate conditions to result in a zero order rate of absorption. This order rate was approached following prolonged accumulation of Rb^+ in the present study and has been reported by Olsen (18) for intact plants under conditions in which a steady state of absorption presumably obtained.

Abrupt changes in concentration result in relatively rapid changes in uptake rates. A possible interpretation is that such changes result first in a new adsorption equilibrium in the protoplasm followed by an adjustment in rate of secretion into the vacuole (or passage to inner cells of the disc) (2). Absorption rates are also influenced by internal concentration as indicated by the lowering of accumulation rates and as previously reported by Sutcliffe (32). The rates found here are not inconsistent with the supposed reactions:

(1) $Rb^+_{out} + HR \rightleftharpoons RbR + H^+$ (adsorptive)

(2) $RbR \rightleftharpoons Rb^+_{in} + R^-$ (absorptive)

where R is a substance binding Rb^+ in uptake and releasing the cation in the cell interior in a subsequent step of the absorptive process. The Freundlich equation is reported to better fit the cases in which the adsorbent is impure or in which a multimolecular layer is adsorbed, whereas the Langmuir equation better fits the cases having a unimolecular layer (7).[3] Since the present results with potato discs show conformance of Rb^+ uptake to the Freundlich equation, it appears that equation (1) above would be better expressed as non-stoichiometric and not necessarily as representing an ion exchange.

(3) $xRb^+ + R \rightleftharpoons Rb_xR$

where R may attract an indefinite number of Rb^+ ions. It should also be pointed out that materials binding Rb^+ may represent more than one substance. This could be true of the initial uptake even though only one substance proved active as a carrier.

[3] Ion uptake has been reported in at least one case (10) to conform to the Langmuir equation. Owing to the relation between the Langmuir equation and the Michaelis-Menten equation (10) other cases of ion uptake conforming to the Langmuir equation appear to exist in published data (8, 9) although not presented in a form designed to show this particular relationship.

SUMMARY

The influence of external concentration of RbCl by the use of tracer Rb^{86} has been studied with respect to the relation of internal Rb concentration during uptake by potato tuber discs. Internal concentration was related to external concentration and conformed well to the Freundlich equation $y = kc^n$, where y is internal concentration, c is external concentration, and k and n are constants.

The amounts of Rb^+ uptake of the initial phase were approximated graphically by extrapolation of uptake curves back to zero time. These values conform very closely to expected (hypothetical) adsorption equilibria.

Approximate values for inward and outward permeability constants (P influx and P efflux) to Rb were calculated from uptake and exchange experiments and on the basis of the approximate area of the exposed cells. The relative low value of permeability outward of labelled Rb^+ in exchange for stable Rb^+ in the external solution seems to be in accord with the idea of a transfer process.

The experimental results are discussed and are believed to be in accord in general with the views as summarized by Arisz (2) and by Helder (10) on uptake and movement of substances in tissue, namely that the protoplasm or symplasm may show an adsorptive relation with the external solution, and that this may limit secretion into the vacuole or limit other consumption, e.g., passage to other cells. The results on uptake rates are not inconsistent with the hypothesis that in accumulation a binding reaction occurs in which Rb^+ combines with a protoplasmic constituent:

$$xRb^+ + R \rightleftharpoons Rb_xR$$

where $Rb\,R \rightleftharpoons Rb^+ + R^-$ may limit the rate during the early phase of active accumulation. It is suggested that R in initial uptake may not represent a single substance or a carrier behaving stoichiometrically.

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NITROGEN METABOLISM IN SCENEDESMUS AS AFFECTED BY ENVIRONMENTAL CHANGES[1,2,3]

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In recent years investigations into the physiology of green algae have been stimulated by a recognition of their possible commercial use (6) in addition to their common acceptance as research tools for certain fundamental problems. These physiological studies have shown that green algae have a high nitrogen content. A correspondingly high protein content has been inferred (28). Relatively little attention has been given to the utilization of the absorbed nitrogen or to changes in the nitrogen fractions of cells subjected to a changing environment. Comparisons with higher plants (7, 30) have not been possible because of the necessity for large and frequent samples of algae required for reliable analytical determinations. The development of a mass culture technique (14, 16) has made this feasible. This paper reports an extensive study of the nitrogen metabolism of a representative, fresh-water, green alga, *Scenedesmus obliquus* (Turp.) Kütz., grown in mass culture. Changes in the content of the major nitrogen fractions are correlated with modifications in the environment, and a basis is established for future detailed examination of specific pathways of nitrogen metabolism.

MATERIALS AND METHODS

The strain, *Scenedesmus obliquus* WH-50, was originally isolated from cultures at the Marine Biological Laboratory at Woods Hole in 1950 and has since been maintained on agar stants. For the nitrogen and dark experiments the cells were subcultured in 500-ml Erlenmeyer flasks containing basal nutrient medium and "aerated" with a mixture of 5 % CO_2-in-air. A packed cell volume of 0.10 ml was added to 300 liters of nutrient solution at the beginning of each experiment. As an inoculum for the potassium experiment, an equivalent volume of cells was taken directly from two agar slants. The mass culture obtained at the end of the potassium resupply experiment served for initiation of the study of phosphorus deficiency.

CULTURE CONDITIONS: The mass culture apparatus has been described in detail in a previous paper (16). Briefly stated, the algae were grown in 300-l, glass-covered, polyethylene-lined, plywood vats, having a surface area of 2 m^2 and a depth of 15 cm. Circulation of the culture was provided by stainless steel stirrers mounted in diagonally opposite corners of the vat. Water for the culture was deionized by an ion exchange column. Five percent CO_2-in-air was supplied through a porous carbon pipe at a rate of 50 l/hr. The cultures were illuminated by a battery of fluorescent and incandescent lamps mounted on a reflector. Illuminance averaged 1000 fc and irradiance averaged 0.12 gm cal/cm^2 × sec at the surface of the culture. The temperature of the solution was held between 25 and 29° C by means of thermostatically controlled air conditioners mounted in the wall of the culture room.

The shaking apparatus for small cultures, described by Krauss (15), was used for certain experiments. Cell suspensions were placed in 500-ml Erlenmeyer flasks fitted with a ground glass joint containing an inlet tube through which 5 % CO_2-in-air was bubbled. The flasks were rocked in a 25° C water bath which was illuminated through a glass bottom by fluorescent lamps.

The basal nutrient solution was that developed in this laboratory (16) containing 1.00 gm KNO_3/l, 0.25 gm $MgSO_4 \cdot 7\,H_2O$/l, and 0.25 gm KH_2PO_4/l. Micronutrients were supplied as inner complex salts of ethylenediaminetetraacetic acid (EDTA). One hundred ml stock solutions of the complexes were made containing 7.70 gm EDTA · NaFe; 7.70 gm EDTA · Na_2Mn; 9.35 gm EDTA · Na_2Ca; 7.15 gm EDTA · Na_2Zn; 7.70 gm EDTA · Na_2Cu; and 6.67 gm EDTA · Na_2Co. Sixty ml of the iron stock solution and 30 ml of the stock solutions of the other complexes were added to each 300 l of nutrient solution. This yielded a concentration of 2 ppm of iron and 1 ppm of the other micronutrients in the basal nutrient solution. The pH of this medium was adjusted to 7.0 with KOH.

The cells were harvested for sampling or transfer by pumping the cell suspension through a Sharples centrifuge. Simultaneously the cells were separated and the medium returned to the vat.

Growth was measured by determining the packed cell volume (hereafter referred to as "pcv"). This was accomplished by centrifuging 100 ml of cell suspension in Goetz phosphorus tubes (13). For calculating the weight of cells removed during sampling, the dry weight of cells per 100 ml was determined by transferring the cells to tared aluminum cups and drying at 100° C.

During the experiments a culture was grown to a pcv of 0.10 ml/100 ml. This was the point of optimum yield, as determined by Krauss and Thomas

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(16). This level was maintained for several days by periodic harvesting. The pH was controlled and the nitrogen level was maintained by the addition of HNO_3 to the culture. After analytical samples had been taken at this level, the cells were harvested and resuspended in a second vat containing fresh medium deficient in the element concerned. In the N-deficient solution, a chemically-equivalent amount of KCl was substituted for KNO_3; in the K-deficient solution sodium salts were similarly substituted for potassium salts; and P-deficiency was produced by using KCl instead of KH_2PO_4. Deficient elements were resupplied by adding KNO_3 for nitrogen, KCl for potassium, and K_2HPO_4 for phosphorus. Analytical samples were taken periodically during the periods of deficiency and resupply.

Extraction and Analytical Methods: Centrifuged cell samples were weighed, and a portion suspended in 1 % acetic acid. The remainder was dried in vacuo at 70° C, weighed, and ground in a Wiley mill to pass a 40-mesh screen. Aliquots of the acetic acid suspension were taken for dry weight and total N determinations. The cells in the remainder of the suspension were killed and extracted by heating the suspension for 5 min at 100° C. Following centrifugation, the cells were extracted 3 more times with water at 70° C. The acetic acid and water extracts were combined, filtered, and made to volume. During the nitrogen experiments, the extracts were stored in the refrigerator; in other experiments they were frozen and stored at −21° C. They were thawed just prior to analysis.

Total-N was determined by a micro-Kjeldahl procedure (23) using salicylic acid to include nitrates and a copper sulfate-selenium mixture as a catalyst. Soluble-N was determined by a Kjeldahl analysis of an aliquot of the combined acetic acid and water extract. Protein-N was calculated by difference between the total-N and soluble-N. In the N-deficiency and resupply experiments free amino-N in the combined extract was determined by a manometric ninhydrin procedure (35). In other experiments the more convenient photometric ninhydrin procedure of Troll and Cannon (33) was used. The photometric results were corrected for the presence of free ammonia which yields 25 % as much colored complex as the amino acids. Peptide-N was determined as the increase in amino-N after hydrolysis with 6 *N* HCl at 110° C in sealed tubes. The photometric values were corrected for the presence of amide- and ammonia-N. Ammonia, total amide, and glutamine analyses of the combined extract were carried out using the vacuum distillation and hydrolytic procedures of Pucher et al (22). Ammonia in the distillate was determined by Nesslerization. Basic-N was analyzed by phosphotungstic acid precipitation (34) followed by a Kjeldahl determination. This N was further fractionated in one experiment by an amino-N analysis of the supernatant. The amount of amino-N removed is a measure of the concentration of basic amino acids. Arginine was analyzed by a modified Sakaguchi procedure (19). It was separated and identified by paper chromatography with phenol-water, ethanol-water, and butanol-acetic acid-water as solvents followed by spraying the paper with the Sakaguchi reagents (4). Nitrate was analyzed using a phenoldisulfonic acid procedure (12). Potassium and sodium were determined in ash solutions by flame photometry employing a Beckman DU spectophotometer. Phosphorus was measured in the ash solution by the AOAC colorimetric method (18).

All determinations were made in duplicate and a set of standards was run with each set of unknowns whenever photometric procedures were used. The deviations from the means were no greater than 5 %, unless otherwise noted.

Results and Discussion

The high nitrogen content of green algae (8, 16, 28) has led investigators to infer that the protein level is also high. The rapid uptake of nitrate described previously (16) suggested that nitrate might accumulate. This possibility was tested by analyses of the water and ether extracts of dried algal samples which showed that only 0.03 % of the dry weight or 0.4 % of the total-N was nitrate. Furthermore, analyses of alcohol-extracted dry material showed that 87 % of the total-N was insoluble. Additional hot water extraction reduced this value to 74 %. These values are lower than those obtained with fresh cells using hot 1 % acetic acid followed by hot water as extractants. Apparently drying results in some proteolysis. The analyses of fresh material showed that approximately 90 % of the nitrogen was insoluble. Analyses of fresh samples from a normal culture showed 15 % of the soluble-N was amino-N, 3 % ammonia- and amide-N, 60 % basic-N, and 0.5 % nitrate-N. About one-sixth of the basic fraction was non-amino-N of basic amino acids, i.e., arginine, histidine, lysine, and ornithine. The rest was presumably nucleotide-N although about 2 % of the basic-N or 1 % of the soluble-N was extractable by chloroform and ether. This treatment is generally regarded as specific for alkaloids (9).

Nitrogen Deficiency: Transfer of N-sufficient cells to a medium deficient in nitrogen resulted in a much reduced growth rate. Figure 1 illustrates this effect. The growth rate is expressed in $\log_2$ units per day which is the reciprocal of the doubling time. Growth is markedly reduced during the first 24 hours of deficiency. This early reduction of growth may result from the shock of centrifugation. The slow but continual growth in N-deficient cultures is due to both sharing of nitrogen by new cells and to storage of photosynthate.

Figure 2 shows that the total-N content decreases exponentially as deficiency progresses and levels off after approximately 8 days. This drop could be due to redistribution of nitrogen among newly-formed cells or to excretion of nitrogen into solution. Calculations made from total-N analyses and dry weight determinations indicate that excretion as well as re-

distribution occurred during growth. These results are shown in table I. About 10 % of the cell nitrogen is lost during the first 12 hours but is reabsorbed during the next 12 hours. This loss and uptake is coupled with the decreased growth rate and with variations from the generally exponential character of the N curve. The shift from protein production to fat production reported in *Chlorella pyrenoidosa* (10, 28) probably occurs during this early period. Production of new material may lag until a different complement of enzymes is formed or activated. After this initial period nitrogen is steadily lost from the cells. This nitrogen may be present in solution in an unavailable form or it may be lost as a gas. The latter seems more likely and could be attributed to the amino acid-nitrite reaction, the loss of ammonia at this neutral pH, or the action of denitrifying bacteria. Similar losses by Chlorella cultures have been reported (20) but have not been substantiated (2). Although the data presented here clearly agree with this earlier report of loss, experiments are planned to reinvestigate the phenomenon in additional mass cultures as well as in small-scale pure cultures.

Nitrogen fraction analyses of progressively deficient cells showed that in relation to the drop in total-N, protein-N remained high, representing 90 to 95 % of the total-N. Soluble-N and free amino-N dropped exponentially and remained at a constant, low level after 3 days. Peptide-N and basic-N remained at a high level for 16 hours and then dropped. The combined amide and ammonia fraction and the nitrate fraction were low. Less than 0.1 mg/gm of each of these fractions was present. Nitrate was not detectable after 16 hours of N-deficiency, but traces of ammonia and amide-N were found in all samples. The analyses of peptide-N, free amino-N, and basic-N reflected the changes in total and soluble-N. A significant difference is not apparent in the proportions of the fractions between normal and deficient cells. This stability in the proportions of fractions indicates a constancy of the mechanism of nitrogen metabolism even under unusual conditions.

TABLE I

LOSS OF NITROGEN FROM SCENEDESMUS CELLS PLACED IN A NITROGEN DEFICIENT MEDIUM

DAYS NITROGEN DEFICIENT	DRY WT/CULTURE	DRY WT REMOVED	TOTAL N AS % DRY WEIGHT	TOTAL CELL-N/CULTURE *	N LOST FROM CELLS
	gm	*gm*	*% dry wt*	*gm*	*gm*
0	81.9	...	7.07	5.80	0.00
0.5	89.0	5.4	5.87	5.22	0.58
1.0	97.0	6.7	5.67	5.82	0.00
2.0	122.7	21.3	4.00	5.29	0.51
4.0	154.5	23.9	2.53	4.76	1.04
9.0	196.0	...	1.69	3.93	1.87

* Corrected for sample removal.

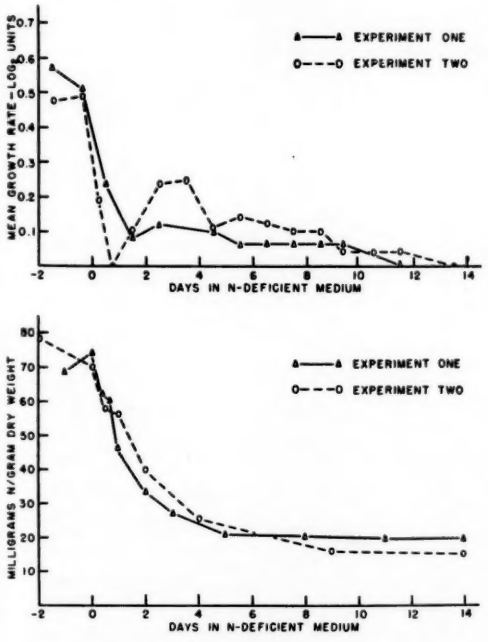


FIG. 1 (*above*). Mean daily growth rate of *Scenedesmus obliquus* during N deficiency as determined from packed cell volume measurements.

FIG. 2 (*below*). Decrease in the concentration of total-N in *Scenedesmus obliquus* cells during N deficiency.

NITROGEN RESUPPLY: After 14 days in the N-deficient medium the cells were resupplied with 55.3 gm KNO_3. This was equivalent to 7.6 gm of N—an amount calculated to bring the level in the cells from 20 to 85 mg N/gm dry weight. After the first 24 hours, an additional 2.2 gm of nitrogen was added in the form of dilute nitric acid which lowered the pH from 8.3 to 7.6.

Upon the addition of nitrate the cells began to grow and a growth rate of 0.7 $\log_2$ unit per day was attained after 24 hours. The cells changed from pale yellow-green color to the deep green characteristic of N-sufficient cultures.

Uptake of nitrogen and protein synthesis are shown in figure 3. There was a lag in uptake and protein synthesis the first 2 hours. The protein level remained proportionally high, protein-N accounting for about 93 to 94 % of the total-N. Presumably during this 2-hr period the metabolic apparatus was being reorganized to handle the increase of nitrogen. During this same period slight readjustments in the soluble fractions occurred as shown in figure 4. After one hour, increases in the basic-N and the amide and ammonia fractions were observed. Analytical difficulties were encountered in analyzing the nitrate and the amide and ammonia fractions, and 5 to 20 % deviations of duplicates from the mean were found. After this initial 2-hr period, all fractions increased rapidly, but nitrate was taken up more rapidly than protein was synthesized. The amount of protein-N

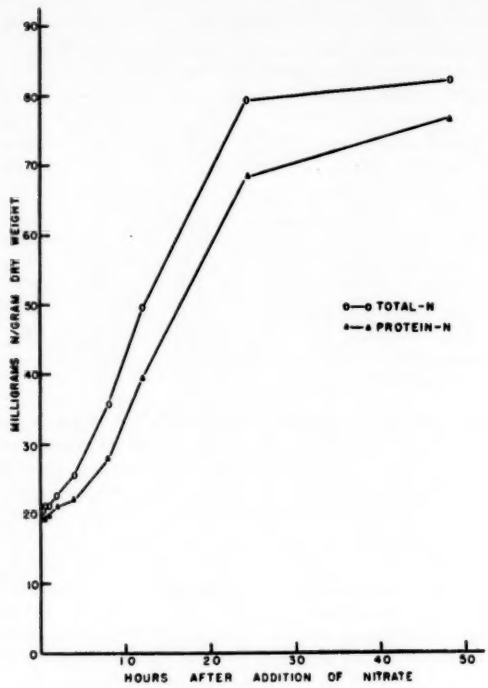


FIG. 3. Increase in the concentrations of total-N and protein-N following the resupply of nitrate to N-deficient *Scenedesmus obliquus* cells.

had dropped to 79 % of the total-N after 4 and 12 hours. Soluble-N accumulated and was mainly basic-N. Smaller amounts of amino-N and peptide-N were formed. Protein synthesis accelerated during the next 12 hours with protein-N accounting for 86 % of the total-N at 24 hours. During the first 12 hours protein synthesis may have been limited by the absence of enough amino acids, peptides and, particularly, basic substances. Alternatively, the accumulation of these substances might be due to some factor which limited protein synthesis. Syrett and Fowden (32) showed that, in the dark, the absence of carbohydrate limited protein formation when ammonia was supplied to N-deficient Chlorella. The present experiments were carried out in the light and pcv values of 0.09 to 0.12 were maintained by sampling. Light intensity per unit cell would be inversely proportional to pcv, and the lowest values were found during the lag of protein formation. Therefore it seems unlikely that light was a limiting factor. After 48 hours protein-N had reached its typical level of 93 % of the total-N. Simultaneously, large decrease in the soluble fractions were observed, and presumably this nitrogen was incorporated into protein.

The composition of the basic fraction precipitated from plant extracts by phosphotungstic acid is extremely heterogeneous and can include basic amino acids, peptides, nucleotides, amines, betaines, and alkaloids. Further fractionation of the basic fraction was carried out by measuring the decrease in amino-N after this precipitation and by analyzing the extract for arginine. These results are shown in table II. During the first 2 hours little change occurred. In all samples taken from 2 to 24 hours most of the basic, non-amino-N consisted of the guanido-N of arginine. However, after 4 hours not all of this nitrogen was in free arginine since multiplying the basic amino-N by 3 yielded results which were about 50 % lower than the guanido-N values. An increase in peptide-N during this period suggested that arginine was combined into a peptide or peptides. This was supported by paper chromatography of the extracts. Using 77 % ethanol as a solvent and a diethylamine atmosphere the unknown had an Rf value of 0.12 while that of arginine was 0.29. Co-chromatography of arginine and the unknown, using butanol-acetic acid, yielded two spots—arginine at Rf 0.20 and the unknown at Rf 0.05. Co-chromatography of the hydrolyzed unknown and arginine gave a single spot at Rf 0.12. The compounds were detected by spraying the Sakaguchi reagents (19) on the paper. Syrett (31) has also reported an increase in arginine upon hydrolysis of similar Chlorella extracts. After 48 hours the arginine content dropped, but the basic-N remained high, suggesting that other basic amino acids had been formed.

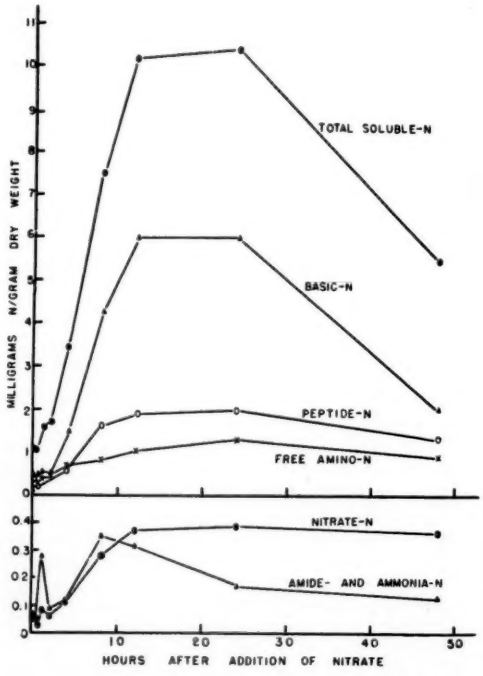
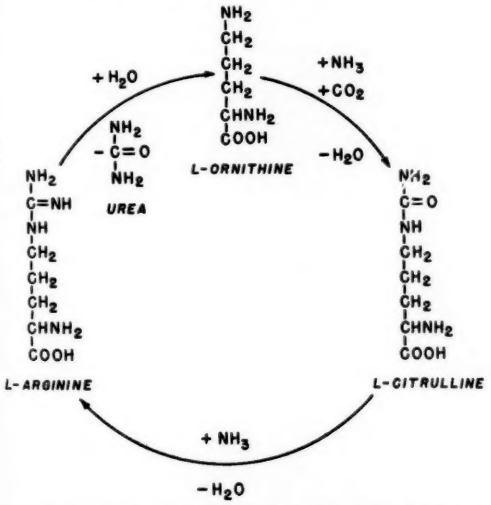


FIG. 4. Changes in soluble-N fractions following the resupply of nitrate to N-deficient *Scenedesmus obliquus* cells.

Under conditions where soluble-N accumulates, one of the responses in higher plants is amide formation (7, 30). Amide and dicarboxylic amino acid formation occurred when young excised pea shoots were supplied with exogenous nitrogen (24), particularly when ammonia rather than nitrate was supplied. Similar results were obtained when N-deficient yeast was resupplied nitrate-N or ammonium-N (26, 36). The rate of protein formation was the same with each nitrogen source, but much more amide and dicarboxylic amino acids were formed in cultures supplied with ammonia than with nitrate. Syrett (31) and Syrett and Fowden (32) showed that amides (particularly glutamine) were formed when N-deficient Chlorella cells were resupplied with ammonia. In contrast to yeast and higher plants, basic amino acids were also formed. The present results using nitrate are similar, but much less amide-N was formed in relation to the other fractions, as was also found with yeast and peas. In the present experiments about 2 hours were required before appreciable accumulation of soluble fractions occurred; responses were immediate in the experiments of Syrett and Fowden. However, the duration of deficiency was only three days in their experiments. Figure 2 shows that in the present experiments a basal level of total-N was not attained before 5 days. Their experiments also show that soluble-N and the fractions thereof decreased after 2 hrs. This could be attributed to the small amount of nitrogen resupplied to their cultures.

An attractive hypothesis concerning the formation of arginine is that the Krebs-Henseleit urea cycle (17) is operative in green algae. This cycle is shown in the following diagram:



An experiment designed to test this hypothesis was carried out using N-deficient cells from the mass culture vats. Harvested cells were suspended in a N-deficient basal nutrient solution, and aliquots of this suspension containing 1.31 gm dry weight were placed in 500 ml Erlenmeyer flasks. To each flask 2.5 millimoles of KNO_3 plus 125 micromoles of a possible precursor were added. The volume was made to 200 ml with N-deficient medium, and the flasks were incubated in the shaking apparatus. Fifty-ml samples taken at periodic intervals were extracted and analyzed for arginine. The results are shown in table III. Stimulation of arginine formation by urea and ornithine was observed after 3 and 6 hrs, but little, if any, stimulation was found at the later times. The lack of stimulation by citrulline may have been due to impermeability of the cells to citrulline as has been shown for liver slices (17). The stimulation by urea could be due to a reversal of the arginase system, since urease is absent from Chlorella (37). Added ornithine would stimulate citrulline formation and a possible reverse reaction involving the enzyme forming citrulline from arginine described in Chlorella by Walker and Myers (38) would in turn result in increased arginine production. In connection with this experiment, the fact that arginine, citrulline, ornithine, and urea are much better sources of nitrogen than ammonium nitrate for the growth of Chlorella (2) is further evidence for the operation of the cycle.

POTASSIUM DEFICIENCY AND RESUPPLY: The mean daily growth rate during 5 days before transfer to deficient medium was 0.334 $\log_2$ unit; during deficiency the rate was 0.119 $\log_2$ unit. The culture was still growing slowly at the end of 21 days in deficient medium. During the first 24 hours soluble-N in-

TABLE II

FRACTIONATION OF THE BASIC NITROGEN FORMED AFTER THE ADDITION OF NITRATE TO NITROGEN-DEFICIENT SCENEDESMUS OBLIQUUS CELLS

HOURS AFTER ADDITION OF NITRATE	MG/GM DRY WT				
	TOTAL BASIC-N	NON-AMINO BASIC-N	GUANIDO-N OF ARGININE	BASIC AMINO-N	AMINO-N OF ARGININE
0	0.46	0.46	0.09	0.00	0.03
1	0.57	0.57	0.11	0.00	0.04
2	0.49	0.32	0.30	0.17	0.10
4	1.46	1.02	1.29	0.44	0.43
8	4.26	3.86	3.45	0.62	1.15
12	5.98	5.03	5.53	0.95	1.84
24	6.03	5.04	4.58	0.99	1.53
48	2.25	1.55	0.27	0.70	0.09

TABLE III

EFFECT OF POSSIBLE PRECURSORS ON THE SYNTHESIS OF ARGININE BY NITROGEN-DEFICIENT SCENEDESMUS OBLIQUUS CELLS

TREATMENT	MICROMOLES ARGININE/GM INITIAL DRY WT			
	3 HRS	6 HRS	9 HRS	12 HRS
KNO_3 control	26	73	100	107
KNO_3 + urea	53	96	109	118
KNO_3 + ornithine	46	90	87	114
KNO_3 + citrulline	28	77	96	111
KCl control	..	..	...	3

creased slightly, but it was incorporated into protein after this time. Throughout deficiency a progressive increase occurred in the proportion of peptide-N from 20 % of the soluble N to 40 % after 21 days. Otherwise no significant shifts in the soluble fractions were observed. After the small initial change protein-N continued to make up most of the total-N. Potassium deficiency in Chlorella causes a decrease in photosynthesis and an increase in respiration (22). Presumably just enough photosynthate was produced in the present experiment to maintain a high protein level, but not enough was formed to enable protein synthesis to continue at a rate sufficient to support normal growth.

After 21 days in deficient medium, the cells were resupplied potassium with 89 gm KCl. The nitrogen fraction analyses during resupply showed that protein synthesis did not start immediately following resupply. A 2 % decrease in protein-N was observed in the first hour. After 3 hrs protein synthesis started and continued throughout the 24 hr experimental period. Similar results were obtained by Pirson (21), who showed that the immediate response to K resupply was an increase in photosynthesis, and that this was followed by an increase in protein as measured indirectly by chlorophyll analyses. In the present experiment, other than an increase in percentage of protein, the only significant change in the nitrogen fractions after resupply was a decrease in peptide-N. The growth rate increased to 1.22 $\log_2$ units per day during the 24 hrs following resupply.

During these studies of the effect of potassium deficiency and resupply on nitrogen metabolism, K and Na analyses were made on ashed cells. The uptake of Na while K was being lost (fig 5) coupled with the continuing growth suggests that sodium may partially replace potassium in the functioning of the alga, perhaps by maintaining electrolyte balance. In barley when Rb or Ca rather than Na were substituted for K, changes in amino acids were much greater (25). It is possible that greater changes in nitrogen fractions of Scenedesmus would occur if some cation other than Na were substituted for K.

Following resupply of K, rapid uptake of K and loss of Na occurred. The usual concentration of K was found after 6 hrs; Na reached a basal level after 3 hrs, but did not drop during the 24 hr period of resupply to the initial low level found before K-deficiency.

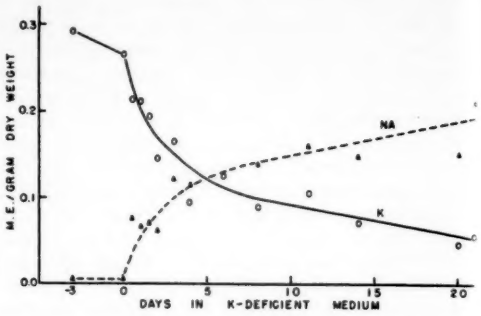


FIG. 5. Changes in the concentrations of K and Na in *Scenedesmus obliquus* cells during potassium deficiency.

PHOSPHORUS DEFICIENCY: Deficiency of P resulted in a significant reduction in growth. The mean growth rate during 3 days prior to deficiency was 0.517 $\log_2$ unit per day, whereas, during 12 days of deficiency the mean growth rate was 0.126 $\log_2$ unit per day. As deficiency progressed the color of the culture became greener, suggestive of phosphorus deficiency symptoms in higher plants.

Nitrogen fractions in deficient cells are shown in table IV. A slight proportional decrease in protein-N was found. In P-sufficient cells 92 % of the total-N was protein-N. This decreased to 87 to 89 % as P deficiency progressed, but the 11-day sample showed recovery. The increase in each soluble fraction generally paralleled the increase in total soluble-N. Similar increases, particularly of amino-N, have been observed in higher plants (29). However, small increases were observed in the proportion of basic-N, expressed as a percentage of the soluble-N, as well as a small relative decrease in free amino-N.

The behavior of the amide and ammonia fraction in early P deficiency was striking, and further frac-

TABLE IV

NITROGEN FRACTION ANALYSES OF PHOSPHORUS-DEFICIENT SCENEDESMUS OBLIQUUS CELLS

DAYS DEFICIENT	MG/GM DRY WT					
	PROTEIN-N	SOLUBLE-N	FREE AMINO-N	PEPTIDE-N	BASIC-N	AMIDE AND AMMONIA-N
0.0	89.3 (92) *	8.28	1.57	1.84	2.86	0.20
0.25	87.4 (90)	9.37	1.93	2.18	5.32	0.32
0.5	84.4 (89)	9.88	2.04	1.70	3.20	1.44
1.0	85.6 (88)	11.65	2.02	2.38	5.88	0.37
2.0	80.2 (87)	11.42	1.76	2.85	5.50	0.32
3.0	77.0 (88)	9.92	1.21	3.94	5.53	0.32
5.0	80.1 (89)	10.24	1.68	2.26	5.32	0.25
9.0	79.8 (89)	10.24	1.55	2.72	5.27	0.41
11.0	75.1 (92)	6.76	1.08	1.52	3.20	0.20

* The numbers in parentheses refer to the protein-N as a percentage of the total-N.

TABLE V

NITROGEN FRACTION ANALYSES OF PHOSPHORUS-DEFICIENT SCENEDESMUS OBLIQUUS CELLS FOLLOWING THE RESUPPLY OF PHOSPHATE TO THE CULTURES

HRS AFTER ADDITION OF PHOSPHATE	MG/GM DRY WT					
	PROTEIN-N	SOLUBLE-N	FREE AMINO-N	PEPTIDE-N	BASIC-N	AMIDE AND AMMONIA-N
0	75.1 (92) *	6.76	1.08	1.52	3.20	0.20
0.5	73.1 (92)	6.89	1.38	2.30	3.15	0.19
1	73.1 (92)	6.34	1.35	0.86	3.10	0.18
3	70.2 (93)	6.12	1.19	0.42	2.79	0.16
8	67.8 (94)	4.64	0.94	0.92	2.35	0.14
18	77.5 (94)	5.07	1.30	1.93	2.43	0.17
30	80.7 (94)	4.88	1.53	1.21	2.45	0.19

* The numbers in parentheses refer to the protein-N as a percentage of the total-N.

tionation into free ammonia-N, unstable amide-N, and stable amide-N was carried out using differential hydrolysis techniques (22). Inasmuch as parallel changes occurred in the basic fraction, analyses for arginine were also made. The results are shown in figure 6. The first effect of deficiency was the formation of basic substances. However, arginine accounts for only approximately 30 % of the basic-N. During the first six hours amides and ammonia also increased slightly. These changes were followed by a large increase in ammonia and in stable amide (presumably asparagine). Preliminary experiments had indicated that most of the amide fraction was unstable (presumably glutamine). In this experiment unstable amide was much decreased. After 12 hours arginine again increased and asparagine and ammonia decreased. These changes could be interpreted as follows: Early in deficiency arginine increases due to the incorporation of ammonia derived from protein breakdown, into the Krebs-Henseleit cycle. After 6 hours soluble-P is used up, and the generation of adenosine triphosphate (ATP) via oxidative metabolism ceases. Gest and Kamen (11) showed that 5 washings with fresh medium removed 25 % of labeled P from Chlorella and that the exchange took place between acid-soluble-P in the cells and exogenous phosphate. ATP is necessary for the operation of the Krebs-Henseleit cycle (17) and for the formation of glutamine (27). Without ATP, ammonia would increase as observed. If the increase of stable amide is an increase in asparagine, these results support a tentative hypothesis that ATP is unnecessary for its formation. However, amide synthesis from ammonia and the corresponding amino acid requires 3450 cal/mole free energy (5).[5] Alternatively, the stable amide could be urea derived from arginine. It would be measured as a stable amide by the hydrolytic analytical method used (22). The increase of arginine and decrease of ammonia and stable amide after 12 hrs could then be due to an increased supply of soluble-P from decomposition of phosphoproteins or nucleic acids. These changes support the concept of a readjusting metabolism when conditions are radically changed.

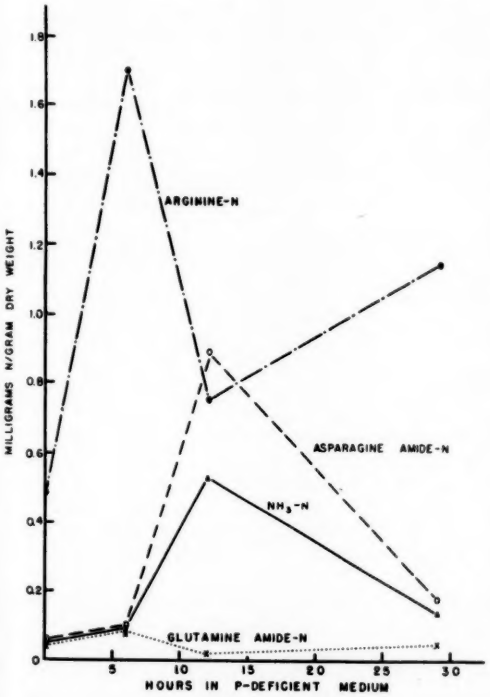


FIG. 6. Changes in cellular ammonia, asparagine, glutamine, and arginine during the early phase of P deficiency in *Scenedesmus obliquus*.

Depletion of total cellular phosphorus was gradual, dropping from 8 mg/gm dry weight to 4 mg/gm dry weight in 11 days. Presumably most of the P is combined in insoluble forms, since appreciable loss does not occur before one day of deficiency. This does not

[5] Recent enzymatic studies (39) have established an ATP requirement for asparagine formation, both for synthesis from aspartic acid and ammonia, and for synthesis by amidation of β-alanine followed by carboxylation of the β-ananylamide. It therefore seems likely that the increase in stable amide is not due to asparagine formation.

TABLE VI

NITROGEN FRACTION ANALYSES OF DARK-CULTURED SCENEDESMUS OBLIQUUS CELLS

DAYS IN DARKNESS	MG/GM DRY WT					
	PROTEIN-N	SOLUBLE-N	FREE AMINO-N	PEPTIDE-N	BASIC-N	AMIDE AND AMMONIA-N
−1.0	89.5 (95) *	4.35	1.45	1.91	2.01	0.19
0.0	88.7 (95)	4.66	1.51	1.28	1.67	0.15
0.3	89.5 (95)	4.95	1.03	1.53	1.63	0.17
0.7	94.3 (96)	4.30	1.04	0.86	1.73	0.13
1.0	90.4 (94)	5.62	1.59	1.46	2.53	0.21
1.5	90.1 (94)	5.40	1.51	1.29	2.61	0.22
2.0	102.1 (93)	7.19	1.46	2.20	3.04	0.23
3.0	96.2 (94)	6.29	1.74	1.67	2.88	0.25
6.0	94.1 (92)	9.45	2.52	2.85	4.42	0.41
10.0	86.8 (91)	9.05	2.34	1.87	3.57	0.87
13.0	85.1 (90)	9.87	1.26	3.67	4.14	0.87

* The numbers in parentheses refer to the protein-N as a percentage of the total-N.

mean that the radical changes in soluble-P, postulated in relation to changes in nitrogenous compounds, do not occur. The changes may happen more rapidly than indicated by total-P analyses.

PHOSPHORUS RESUPPLY: After 11 days of deficiency 48 gm K_2HPO_4 was added to the culture. This supplied approximately half as much P as was initially supplied in a P-sufficient medium. Rapid growth of the cells resumed; a rate of 0.690 log unit per day during the first 24 hrs was observed. Changes in the nitrogen fractions are shown in table V. Steady resynthesis of protein occurred. Ninety-two percent of the total-N was protein-N initially, and this increased to 94 %. A concomitant decrease in soluble-N was found. The assumption is made that as soluble-P became available ATP necessary for protein synthesis increased. The proportion of amino-N increased from its previously low percentage of the soluble-N. Changes in basic-N followed the changes in soluble-N. Amide and ammonia-N did not change. Peptides increased and then decreased, suggesting a role as intermediates in protein formation.

The changes in total cell phosphorus were rapid. Phosphorus reached the usual level during the first hour; almost doubled in the next 8 hrs; and then decreased to an equilibrium value. Luxury consumption clearly occurred during the early period.

DARK EXPERIMENT: A mass culture was grown for 6 days in the light. Then the lights were turned out and the algae were cultured for 12 days in the dark. The mean growth rate during the light period was 0.584 $\log_2$ unit per day. Apparent growth rates as calculated by pcv measurements were 0.325, 0.328, and 0.186 during the respective first 3 days in the dark. After this period no growth was observed.

Changes in the nitrogen fractions during dark culture are shown in table VI. An increase in total-N was observed at the second day and was followed by a decrease. The proportional amount of protein-N remained high until 3 days and was followed by a decrease. The high levels of total-N could be accounted for by the utilization of non-nitrogenous reserves by the respiratory system. Protein breakdown occurred after these reserves were exhausted and protein carbon was utilized to provide energy for the cell. Soluble-N was then formed. Proportions of the soluble fractions remained more or less constant until after the tenth day when the proportion of amino-N decreased. At this time amino acids were being deaminated and respired, thus accounting for the increase in the amide and ammonia fraction. When the experiment was terminated on the 13th day, 1.58 mg NH_3-N/l was found in the culture solution, indicating that some excretion had occurred. Only traces of amino-N were found in the solution. In spite of the increase in soluble-N and changes in the fractions, it should be emphasized that the change in protein-N was small and that protein-N accounted for 90 % of the total-N at the end of the experiment.

CONCLUSIONS

All of the factors studied, i.e. nitrogen, phosphorus, potassium, and carbohydrates, are essential for protein synthesis in *Scenedesmus obliquus*. Growth was much reduced or ceased if any one of these was deficient. A striking fact is that only one of these, exogenous nitrogen, was required for the maintenance of a high protein content. The absence of other factors resulted in only a limited amount of proteolysis. Even during nitrogen deficiency, the bulk of the nitrogen in the cell was protein-N. Thus the mechanism for protein formation is not damaged by any of the deficiencies studied. Overflow or shunt metabolism occurs in Scenedesmus during nitrogen deficiency. Carbon which ordinarily would be incorporated into protein was formed into fat and carbohydrate (1). However, the present studies show that Scenedesmus is predominately a protein-forming organism.

During the first 24 hours of deficiency of any of the factors slight readjustments of nitrogen metabolism occur. In the case of phosphorus, these changes could be correlated with possible changes in phosphorus-containing components of the cell. In nitrogen deficiency, excretion and reuptake occurred. Potas-

sium deficiency resulted in an accumulation of soluble-N fractions without changes in their proportions, and this was followed by a decrease and reincorporation into protein. Darkness resulted in slight changes of the nitrogen fractions.

Following resupply of N, P, or K, protein synthesis resumed. In the case of P, synthesis was resumed almost immediately. Metabolic readjustment occurred during the first few hours following resupply of K and presumably protein synthesis must be preceded by resumption of the photosynthesis. Similar metabolic readjustment occurred when N was resupplied. This was followed, however, by an increase in basic substances, particularly arginine-containing peptides. The accumulation of basic-N may be the result of overloading the protein synthesis system with N so that part of it overflows into the basic fraction. Similar shunt metabolism of P-containing compounds may occur since luxury consumption of P was found during resupply. However, with regard to N, all the experimental data indicate that if alternative pathways exist when cells are subjected to peculiar conditions, they are characterized by a low synthetic capacity which prevents major accumulation of any N-fraction other than protein.

Summary

Scenedesmus obliquus, strain WH-50, was grown in mass culture under conditions of N, P, K, and carbohydrate deficiency. Nitrogen, P, and K were resupplied to the cells. During the experiments samples of cells were harvested and analyzed for nitrogen fractions.

In these experiments, the following results were obtained:

(1) During N deficiency the N content of the cells dropped exponentially and reached a basal level after 8 days. This decrease in total-N was due partially to dilution by new cell material and in part to the excretion of N from the cells. During N deficiency the relative proportions of the fractions were little changed. Protein-N continued to account for approximately 90 to 95 % of the total-N.

(2) Upon resupplying nitrate to N-deficient cells a normal growth rate was resumed during the first 24 hrs. Protein synthesis lagged behind N uptake and soluble-N accumulated in the cells. Most of the soluble-N consisted of basic-N; and most of this was tentatively identified as arginine-containing peptides. Precursor experiments indicated that the synthesis of arginine probably occurred through a Krebs-Henseleit urea cycle similar to that found in liver.

(3) Potassium deficiency resulted in a reduction in the growth rate of the culture, but growth never ceased during a 21-day K-deficient period. No appreciable shifts in the nitrogenous composition occurred other than an initial small decrease in the protein-N and an increase in the level of peptide-N. The K level in the cells slowly dropped and was replaced by Na. Resupply of K to K-deficient cells resulted in a resumption of normal growth, a decrease in the amount of peptide-N in the cells, and a resumption of protein synthesis. The cells contained a normal amount of K after 6 hours, and a concomitant loss of Na occurred.

(4) During P deficiency the amount of protein-N decreased only slightly. Early in the deficient period, changes in free ammonia, stable and unstable amides, and in arginine occurred. The resupply of P to deficient cells enabled the culture to resume normal growth. Steady protein synthesis followed resupply. Except for changes in the peptide fraction, most of the soluble fractions paralleled the behavior of the soluble-N.

(5) In darkness an increase in total-N occurred as non-nitrogenous reserves were utilized in respiration. After 2 days in the dark, the total-N decreased. As protein was broken down increased proportions of soluble-N were found. After 13 days of darkness, amino-N had decreased and the amide and ammonia fraction increased. Apparently deamination of amino acids had occurred, for ammonia-N also was found in the culture solution. However, little proteolysis occurred.

It is concluded that while adequate supplies of N, K, P, and carbohydrate are essential for protein synthesis, only N is required for the maintenance of protein once it is formed. Although metabolic readjustments and shunt metabolism do occur under unusual circumstances, protein makes up most of the total-N, and the metabolism of the alga is strongly directed toward the formation and maintenance of protein.

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EFFECTS OF MICRONUTRIENT DEFICIENCIES ON MINERAL COMPOSITION, NITROGEN FRACTIONS, ASCORBIC ACID AND BURN OF TOBACCO GROWN TO FLOWERING IN WATER CULTURE[1]

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A systematic examination was undertaken of absorption and accumulation of the biologically essential elements by the tobacco plant when grown to the flowering stage. Both complete and micronutrient deficient cultures were studied. The basal nutrient solution employed was an approximately maximal yield solution of minimal salt content. Its composition had been determined in consecutive tests each spring since that of 1945, using both increased and decreased quantities of individual salts to determine maximum possible yields with minimal supply of salts. The experimental installation was kept unchanged during this time and a uniform procedure used in handling seedlings. Its composition could be assumed to have meaning only for the one variety and environment.

It will be shown that the composition of the basal solution so determined was found to be such as to undergo very little change in relative proportions of its chemical elements as a result of the growth of the plant to the flowering stage. Hoagland (9) has referred to this type of nutrient solution in these words —"There is another concept which may be employed in the selection of salt proportions for a nutrient solution—that of what may be called 'economical' utilization of nutrients. By this is meant the preparation of a solution from which the plant will absorb the various ions in the same proportions as they are originally present in the solution." It would follow therefore that the relative proportions of essential elements in the plant as a whole should be the same as those originally present in this type of solution.

The effects of micronutrient deficiency in the basal solution were also studied. Omission of individual micronutrients led only to minimal changes in initial composition of the nutrient solution. A difficulty existed in that deficiencies of equal severity on a yield basis could not be produced at extreme levels. Such differences in severity, however, were taken into account in interpretation of the results, and served as an aid in distinguishing between general and specific responses to deficiency of the individual micronutrients.

The fixed-charge depletion technique (15) was used in conjunction with calcium carbonate purification of the nutrient solution to remove trace element impurities (17). Only distilled water (i.e., condensed heating steam) was replenished during growth of the plant. Absorption was maintained by periodic stirring of the nutrient solution to keep newly formed root-hair surfaces covered with a layer of the precipitated salts. Wide fluctuations in initial acidity (pH 4.6 to 7.2) in such cultures had been found to be without perceptible influence at the 5% level of variance on yields or the optima for calcium and magnesium.

These results were in full accord with those reported by Hoagland and his co-workers for many crop plants. Hoagland (9) concluded ". . . There seems to be a relatively wide range of hydrogen ion concentrations over which this factor *per se* has no highly significant effect on the plant." A similar conclusion was arrived at by Olsen (13) with respect to ion intake.

This does not mean that secondary effects such as precipitation of iron at high pH cannot affect growth. It does mean that if, for example, stirring of solution to deposit precipitated iron salts on the root-hair surfaces can be used to maintain intake, the high pH would not be found deleterious for growth. Colloidal iron, in this case, was found to be as effective as various sources of soluble iron when stirring was employed.

The biochemical changes in nitrogen fractions of the leaves were also followed in these experiments (16). Preliminary surveys were included with respect to ascorbic acid content and burn of the leaves. The leaf content of mineral and nitrogen fractions are reported as 2-year averages, both for spring and fall growth with the same solution. This is to say no special basal solution specifically adapted for fall use was employed but the spring basal solution was used at both seasons of the year. Interpretations are based primarily on results in spring; and the data for fall included only for comparison and check.

EXPERIMENTAL METHODS

Single plants of Connecticut Broadleaf tobacco were grown in triplicate to the flowering stage in Pyrex jars containing ten liters of a maximal yield with minimal salt supply solution adjusted for spring growth. Temperature in the greenhouse was maintained at about 25° C both day and night. Details of the cultural and purification ($CaCO_3$) processes to minimize trace element contaminations will be found in previous publications (15, 17). Duration of growth in the jars approximated fifty days. Air-dried weights were obtained by separating the tops and roots on harvest and hanging them to dry in the greenhouse. About three months were required. Yields were recorded as leaf, stalk, root and total. Where necessary, corrections were applied for loss in yield by sampling of the plants. All data herein are considered

[1] Received August 4, 1954.

directly comparable though obtained to some extent in consecutive years.

The absorption data and average mineral content for the plants as a whole were obtained in single experiments in spring and fall. The solutions and not the plants were analyzed. Equal volumes of solution were taken from each set of triplicates after making up to volume and were then mixed for assay. Computations for the probable average mineral contents of the plants as a whole were made from the absorption and air-dried yield data.

Leaf samples for mineral analysis consisted of separate samples at top, middle and bottom of the stalks. Dead tissue was avoided in sampling. The detached leaves were immediately dried in a current of air at 80° C for about three hours. The values for the above three samples have been averaged in this report to conserve space. Comparison runs at various times consisted of individual samples from each triplicate plant, mixed samples of the triplicates, early and late sampling, leaf lamina only, total micronutrient levels, and dilute as compared to standard concentration nutrient solution. While these latter data are also not included in this report they have been of aid in interpretation. The dried samples were ground to 60-mesh and bottled. The reported data are two-year averages for spring and for fall.

Leaf samples for estimation of nitrogen fractions were handled in a similar manner. Three middle leaves of each triplicate plant, however, were combined into a single sample after drying. These determinations also consisted of four experiments; the values for spring and fall being two-year averages.

The ascorbic acid determinations were made by the method of Loeffler & Ponting (10) on combined samples of freshly picked leaves (8:00 to 9:00 A.M.) by Dr. Boyce D. Ezell and Miss Marguerite S. Wilcox. Analysis was made immediately after sampling, the midribs being discarded. Three series of spring samples were assayed in separate years. The burn data for air-cured leaf (duration of glow without flame) are the average of several experiments.

Calcium, Mg, P, Na, B, Al, Fe, Mn, and Cu were determined with a Bausch and Lomb large Littrow quartz spectrograph using a D.C. arc burning at 150 volts and 24 amperes (1). Potassium was determined using a Beckman spectrophotometer with flame attachment. The data on Na and Al are not discussed, since they are not considered essential for tobacco.

Total nitrogen assays were by Kjeldahl (4, p. 13). Ammonia plus organic nitrogen was estimated with a modified Kjeldahl method (4, p. 12); and acid coagulated nitrogen also by Kjeldahl (3, p. 281). The difference between total nitrogen and ammonia plus organic nitrogen was assumed to represent nitrate nitrogen. Soluble organic nitrogen was obtained by subtracting acid coagulated nitrogen from organic nitrogen.

In the above estimations soluble organic nitrogen is assumed to approximate the free amino acid fraction, and acid coagulated nitrogen, the protein fraction. Comparison of nitrogen fractions have been

TABLE I

MINERAL COMPOSITION OF ENTIRE, FLOWERING, CONNECTICUT BROADLEAF TOBACCO PLANTS GROWN IN MICRONUTRIENT DEFICIENT SOLUTIONS

CULTURE SOLUTIONS	TOTAL YIELD, GM	COMPUTED COMPOSITION OF AIR-DRIED PLANTS *										
		MG/GM						μG/GM				
		N	P	K	Ca	Mg	Fe	Cu	Mn	B	Na	Al
						Spring						
Initial **	....	15.2	6.0	16.3	28.0	6.0	1.51	0.7	65	62	50	9
Final	100.5	14.8	5.0	16.1	24.5	6.0	1.44	0.8	62	60	33	5
- Mo	93.5	16.1	5.7	17.4	26.1	6.4	1.12	0.9	68	54	44	6
- Fe (0.5)	63.9	23.7	6.6	25.4	35.2	9.4	0.06	4.9	97	92	63	...
- B	55.4	27.1	7.4	29.3	45.1	10.8	2.12	0.5	104	39	70	6
- Cu	44.0	29.5	7.5	36.9	47.7	10.9	2.44	...	114	127	89	2
- Zn	32.0	29.1	17.7	50.6	60.9	11.6	3.94	2.5	159	150	100	...
- Mn	9.8	12.4	19.4	89.8	96.9	12.2	9.31	3.1	0	449	174	...
						Fall						
Initial **	....	19.8	8.5	17.7	30.0	4.6	1.51	0.8	65	49	40	6
Final	63.9	30.8	8.8	27.6	34.4	7.2	1.37	...	85	66	24	...
- Cu	46.2	42.1	13.2	38.3	49.8	9.9	2.15	2.2	129	97	50	1
- Mo	45.7	41.9	11.2	38.6	46.0	9.4	1.87	8.3	116	79	50	...
- B	38.6	45.7	12.2	45.8	46.6	9.8	2.11	6.7	150	19	52	...
- Fe (0.2)	29.7	41.5	11.8	59.5	43.8	9.1	0.06	20.5	219	131	67	...
- Zn	25.5	36.4	21.5	58.9	51.0	11.0	3.43	14.1	242	114	35	...
- Mn	8.8	6.9	28.4	113.6	90.9	22.7	8.35	56.8	0	309	125	2

* Absorption from nutrient solution in mg or μg/total air-dried wt of plant in gm.

** Actual concentration × 100. A slightly better agreement between solution and control values would result from the use of 98 as a factor.

made both on the basis of dry matter and of organic nitrogen present in the leaf tissues, in view of the differences in viewpoint among investigators.

ABSORPTION OF CHEMICAL ELEMENTS: The results obtained in the absorption experiments for spring and fall growth are shown in table I. The values were computed from total air-dried yields and quantities of element absorbed from the nutrient solution. No determinations of S, Zn and Mo are included. These three elements were added to the nutrient solution as 21.6, 0.2 and 0.05 ppm, respectively. They represented about 0.09 % of the mg of essential elements supplied. Absence of analyses for these three elements could not have seriously affected the relationships discussed in this manuscript.

The basal nutrient solution differed somewhat in composition each time it was prepared because of the need for purification to remove trace element contamination. The top rows give the quantities of each element times 100 found in the solution before growth of the plant. Of the amounts shown as present in the complete basal solution the following quantities were added after purification: Fe, 15.0; Zn, 0.2; Cu, 0.05; Mn, 0.65; B, 0.35 and Mo, 0.05 ppm. Deficient solutions had minimum values for the elements withheld.

It will be noted that the computed values for composition of the plants, whether normal or suffering from a micronutrient deficiency were perceptibly increased when plants were grown in fall. In almost every case the content of each essential element in the plant was higher with plants grown in the fall than in the spring.

The effects of micronutrient deficiencies on computed composition of the plants showed good agreement for both seasons of growth. Deficiencies in the table were arranged in order of diminishing yield of the plants. It can be seen that some elements, as P, K, Ca and Mg, show a steady rise in concentration in the plant with decreasing growth caused by miscellaneous micronutrient deficiencies. These variations irrespective of type of deficiency are considered non-specific and associated only with growth as a whole. A value such as 4.9 for Cu in the spring when Fe is almost absent from the nutrient solution is out of line with the general trend and thus would appear to indicate a specific effect caused by Fe deficiency. The fall value for Cu (20.5) would tend to confirm this interpretation based on trends in composition with changing yields.

Agreement in relative proportions of the essential elements of the freshly prepared complete solution and the computed values of the control plants on harvest was excellent for the spring experiment (table II). The quantities differed by less than ± 10 % from the averages of the two series of determinations. Use of a similarly prepared spring basal solution in fall led to much poorer results (about ± 20 %) apparently because it had not been adjusted in composition specifically for fall use. Agreement in both seasons was sufficiently close to indicate that a maximal yield solution of minimal salt content does not change in relative proportions of ions as a result of the growth of the plant. It can be computed from values in table I that total absorption of elements from the complete nutrient solutions approximated 93.2 and 85.7 % for the spring and the fall runs, respectively (Na and Al omitted from the calculation).

TABLE II

VARIATIONS BETWEEN INITIAL COMPOSITION OF THE COMPLETE NUTRIENT SOLUTION AND THE COMPUTED COMPOSITION OF WHOLE FLOWERING PLANTS OF CONNECTICUT BROADLEAF TOBACCO

ELEMENT	SPRING *		FALL *	
	AVE **	± VAR	AVE **	± VAR
		mg		
N	15.04	0.24	25.32	5.48
P	5.49	0.51	8.63	0.13
K	16.20	0.10	22.67	4.97
Ca	26.24	1.76	32.22	2.22
Mg	5.99	0.02	5.88	1.28
Fe	1.48	0.04	1.44	0.07
		μg		
Cu	0.77	0.04	0.78	...
Mn	63.60	0.14	74.80	9.80
B	60.75	1.25	57.40	8.40

* Composition of the complete nutrient solution was adjusted for maximum yield of plants with minimal supply of salts during spring, *not for fall.*

** Solution × 100 and plant per gm.

The results of absorption tests in table II for spring grown plants illustrate the degree of precision attained. The greatest variation in relative compositions between plant and solution was in the case of phosphorus, which varied by ± 9.3 % from the mean of both series being compared. Calcium and Cu variations were also high, being ± 6.7 and ± 5.2 %, respectively. Variations in relative content between plant and solution for the other six elements were well below ± 5 %: Mg ± 0.35 %, Mn ± 0.22 %, and K ± 1.23 %. Nitrogen varied by ± 1.59 %. While greater precision might have been attained through chemical methods of analysis and full statistical procedures, the precision used was ample for the purpose in mind.

The values for leaf content (table III of the essential chemical elements were determined directly with leaf samples. Regularities in trend for changes in yield were much poorer in the case of leaves than for the whole plant. It would seem that micronutrient deficiencies affect leaf composition in a specific manner far more than that of the plant as a whole. Note, for example, the very high values in both seasons for Mn content of leaves when Fe is deficient and for Fe and phosphate when Zn is deficient. The leaves of the control plants in spring appeared to be higher in N and Cu content than that in the plant as a whole, whereas K, Ca, and Mn were at the same levels. Phosphorus, Mg, Fe and B were somewhat lower in concentration in the leaves of the spring control plants than in the plant as a whole.

TABLE III

ESSENTIAL ELEMENTS IN LEAF OF FLOWERING, CONNECTICUT BROADLEAF TOBACCO PLANTS GROWN IN MICRONUTRIENT DEFICIENT CULTURES

CULTURE SOLUTIONS	DRIED LEAF, GM	MINERAL CONTENT OF LEAVES										
		MG/GM						μG/GM				
		N	P	K	Ca	Mg	Fe	Cu	Mn	B	Na	Al
						Spring						
Complete	55.8	22.3	3.6	14.8	23.4	1.9	0.14	6.0	72	38	24	186
– Mo	41.8	23.1	3.8	13.6	30.0	2.4	0.12	8.2	94	50	26	248
– Cu	28.8	57.2	6.9	27.7	48.2	5.6	0.26	3.3	134	100	26	246
– B	28.8	37.3	3.4	28.8	50.5	4.6	0.13	6.4	132	16	27	173
– Fe (0.35)	22.8	53.7	4.9	21.8	35.0	4.7	0.12	11.4	170	63	21	247
– Zn	19.2	51.2	16.3	38.0	55.8	6.8	1.09	14.6	243	168	23	303
– Mn	8.8	71.5	6.1	88.3	33.0	5.3	0.22	36.4	9	161	35	292
						Fall						
Complete	27.9	52.8	5.7	24.7	41.7	7.1	0.11	7.3	99	36	18	46
– B	28.1	47.7	4.4	37.2	41.7	5.5	0.16	7.6	138	11	21	128
– Cu	23.7	61.2	8.2	32.5	49.8	8.5	0.14	2.4	123	38	17	63
– Mo	22.8	66.9	9.3	31.0	56.0	8.0	0.13	7.3	129	47	18	88
– Zn	17.7	51.8	17.3	54.3	46.2	5.2	1.11	10.8	194	48	17	37
– Fe (0.2)	10.9	58.8	7.4	64.7	45.2	6.6	0.08	22.0	216	56	26	34
– Mn	7.3	77.1	6.9	106.3	32.7	4.5	0.14	33.5	6	49	26	58

Nevertheless the same seasonal difference found between spring and fall grown plants in composition of the plants as a whole are reflected in leaf composition for the two seasons. This is shown in the cases of the control plants and of plants suffering from each micronutrient deficiency.

Table IV presents the results for nitrogen fractions in the leaf. They have been computed both on the basis of percentage of dry weight and as percentage of organic nitrogen. The values for both seasons of the year are two year averages, and tend to confirm each other. Appended to the spring results are the values obtained for the leaf samples from control plants grown on the complete solution for two consecutive years. The percentage errors on the dry weight basis were less than 0.12 %. On a percentage organic nitrogen basis the values differed by not more than 4.7 %. The micronutrient deficiencies have been arranged in the order of decreasing leaf yields.

Most of the values on a dry-weight basis for spring grown plants increased regularly with decreased yields except nitrate-nitrogen with Mo deficiency and soluble

TABLE IV

TWO-YEAR AVERAGE FOR NITROGEN FRACTIONS IN LEAVES OF FLOWERING CONNECTICUT BROADLEAF TOBACCO GROWN IN MICRONUTRIENT DEFICIENT CULTURES

CULTURE SOLUTIONS	% NITROGEN					AS % ORGANIC N			TOTAL WT OF LEAF, GM	NITRATE N IN LEAF, MG
	TOTAL	ORGANIC	NITRATE	SOLUBLE ORGANIC	COAGULATED	SOLUBLE ORGANIC	COAGULATED	NITRATE		
					Spring					
Complete	2.27	2.18	0.10	0.56	1.62	25.7	74.3	4.4	53.6	53.6
– Mo	3.91	3.53	0.38	1.57	1.96	44.5	55.5	10.8	38.6	146.7
– Fe (0.2)	4.20	3.97	0.23	1.78	2.19	44.8	55.2	5.8	37.3	85.8
– B	4.43	4.26	0.17	2.16	2.10	50.7	49.3	4.0	36.3	61.7
– Cu	5.06	4.92	0.14	1.17	3.75	23.8	76.2	2.8	34.5	48.3
– Zn	5.71	5.35	0.36	2.98	2.37	55.7	44.3	6.7	25.2	90.7
– Mn	7.63	6.58	1.05	3.63	2.95	55.2	44.8	16.0	5.6	48.3
Complete,										
1952	2.23	2.14	0.09	0.50	1.64	23.4	76.6	4.2	52.0	46.8
1953	2.31	2.21	0.10	0.62	1.59	28.1	71.9	4.5	55.2	55.2
					Fall					
Complete	5.28	5.10	0.18	1.90	3.20	37.3	62.7	3.5	32.3	58.1
– B	4.77	4.40	0.37	1.73	2.67	39.3	60.7	8.4	27.4	101.4
– Mo	6.99	5.01	1.68	2.67	2.34	53.3	46.7	33.5	25.2	423.4
– Cu	6.12	5.73	0.39	2.55	3.18	44.5	55.5	6.8	20.6	80.3
– Zn	5.18	4.56	0.62	2.55	2.01	55.9	44.1	13.6	19.6	121.7
– Fe	5.88	5.29	0.59	2.04	3.25	38.6	61.4	11.2	10.8	63.7
– Mn	7.71	6.17	1.54	3.14	3.03	50.9	49.1	25.0	7.6	117.0

and coagulated N with Cu deficiency. This inverse relation to yield was also evident to a lesser degree in percentage of nitrate-nitrogen and soluble nitrogen in fall grown plants. Nitrate-nitrogen with Mo deficiency was again exceptionally high when compared on a loss in yield basis in the fall experiments.

It will be noted that on a dry-weight basis nitrate accumulated in the leaves at both seasons of the year with all types of micronutrient deficiency. All values for nitrogen fractions in the leaves on the dry-weight basis were, almost without exception, higher for fall growth than for spring growth.

On a percentage organic N basis, soluble organic N increased relative to the controls in the case of all deficiencies in spring except that of Cu. Coagulated N decreased relative to that in the controls also except for Cu in spring experiments. Nitrate accumulation relative to that of the controls was also found at both seasons on an organic N basis with all micronutrient deficiencies, except B and Cu in the spring.

Molybdenum deficiency caused outstanding accumulation of nitrate in the leaf at both seasons on both bases. This is clear both from the figures for percent nitrate-N and those for mg nitrate-N in the leaves. Nitrate accumulation apparently increased in reverse relation to yield. The greatest effect on nitrate accumulation was therefore related to Mo deficiency, since yield in the absence of this element was affected only moderately. A decrease in yield of about 90 % was necessary under these conditions with Mn in order to exceed the accumulation of nitrate with partial deficiency of Mo.

Results on ascorbic acid content of the fresh leaf with micronutrient deficiency and the burn in seconds of the air-dried leaf are summarized in table V. The latter is also referred to as "duration of glow" of the leaf when ignited without production of flame.

The ascorbic acid data are regarded as preliminary in nature, since they characteristically fluctuate widely. Large scale tests would be needed to obtain greater uniformity. As will be seen these, however, would be of less value to some extent without control of micronutrient fertilization. Decrease in ascorbic acid content of the leaf lamina seemed to take place whenever the deficiency was severe enough to decrease yield. Iron and Mo deficient plants grown in the spring of 1952 were the only exceptions. In neither of these two exceptions were yields seriously affected, nor leaf symptoms prominent.

Ascorbic acid content was decreased most effectively by deficiencies of B and Mo. This statement is based on the fact that whereas the – Mn plants attained a yield of only about 10 % that of the control, the yield of the – B and – Mo plants were about 50 and 80 % that of the control, respectively. The symptoms of Mo deficiency were most severe in the 1954 experiment.

The differences in level of ascorbic acid in the three series are attributed to differences in nitrogen level. That in 1952 was least, and that in 1953 most. The latter was in the range of "luxury" consumption. This interrelation of nitrogen and ascorbic acid levels is known (5, 7).

Effects of micronutrient deficiencies on burn of the leaf are presented at the bottom of table V. Only in the case of Mn and B deficiencies was burn affected to any extent.

Discussion

The results on mineral absorption appear to be of importance in several respects. There is first the fact that the experimental adjustment of a nutrient solution to produce maximum yield with a minimum of salts results in an "economical" solution. That is, net absorption of nutrient constituents by the plant up to the flowering stage is effected without change in proportions of nutrient components in either solution or plant as a whole.

The steady rise in mineral content of the plants as a whole with diminution in yield irrespective of type of deficiency made evident another important factor in mineral nutrition. It follows that plants growing in the same solution and differing to any extent in size cannot be directly compared with each other with respect to absorption. This relation between yield and absorption is also seen between the mineral content of the plants grown in the spring as compared with the smaller plants of the fall. This yield-supply relationship was far from negligible.

Table V

Ascorbic Acid Content and Burn of Lamina of Flowering Connecticut Broadleaf Tobacco Grown in Micronutrient Deficient Cultures

Determination	Year	Cultures						
		Complete	– Fe	– Zn	– Cu	– Mn	– B	– Mo
				Green leaf				
Ascorbic acid (mg/100 gm)	1952	17.1	20.8	12.4	18.5	12.7	5.3	28.3
	1953	65.7	47.5	31.8	50.0	21.5	4.0	16.6
	1954	37.5 *	28.4	21.0	29.0	14.6	10.4	2.9
	Ave., 1952–54	40.1	32.2	21.7	32.5	16.3	6.6	15.9
				Dried leaf				
Burn, sec	1949–52	3.6	2.8	3.7	2.7	24.1	11.9	4.1

* Average for 3 plants assaying 29.5, 41.2 and 41.9 mg ascorbic acid/100 gm fresh leaf lamina.

It was also evident that micronutrient deficiency affected relative mineral composition of the plant as a whole only slightly as compared to that of the leaves. The reason for this may consist of a compensatory decrease in growth of the whole plant with deficiency. Though none of the data obtained are presented herein, the evidence found made clear that decreased micronutrient content was more apparent the closer the sample taken was to the locus of maximum symptoms in the plant. A similar situation had been reported for K and B (18). The small decreases in micronutrient content with deficiency in the leaves were quite striking as compared to those found in the plants as a whole. Distribution within the plants is evidently greatly affected by deficiency in favor of the leaves.

Greater weight should, it is believed, also be placed on the spring than the fall data as concerns nitrogen fractions in the leaf because of poorer light conditions in winter. A greater degree of agreement in spring and fall results was shown when percent organic nitrogen was used as a basis of comparison. The regular rise in nitrogen fraction content of the leaves with decreasing yields is interpreted solely as a non-specific yield-supply response as in the similar case of mineral contents already mentioned. Accompanying these general increases were definite changes in proportion of nitrogen fractions caused by micronutrient deficiencies. Included in this category are the effects of deficiency in causing an increased content of soluble organic nitrogen (free amino acids) and of nitrate; and a decreased content of coagulated nitrogen (protein). These changes are similar to those already reported (16, 18). The differing results with copper deficiency in spring agree with those of Lucas (11).

The outstanding effects of manganese and molybdenum deficiencies on accumulation of nitrate (2, 6, 8, 14) would seem to be associated with decreased enzyme formation (12) in the case of the latter. Hewitt (8) had found nitrate accumulation to be greatest with manganese and molybdenum deficiencies; and that whereas free amino acid content present in the former condition was characterized as high, practically none were present in the latter case. Free amino acids were lower with molybdenum than manganese deficiency in spring as compared to fall. Molybdenum deficiency was presumably insufficient in extent to show any greatly marked decrease in formation of free amino acids.

The significance of the data on ascorbic acid has been discussed in comment on table V. The data would indicate that boron as well as molybdenum deficiency (8) strikingly inhibits the formation of ascorbic acid. The interpretation of Hewitt that ascorbic acid participates specifically in the reduction of nitrate-N therefore would seem to need further verification.

Summary

Connecticut Broadleaf tobacco was grown to the flowering stage during spring and fall in the greenhouse at about 25° C. The fixed-charge, depletion method was used. Each plant was grown in ten liters of a purified nutrient solution adjusted for maximal yield and minimal ion content in spring. The calcium carbonate method was used to remove trace element impurities from the nutrient solution. Yield tests were in triplicate; and analyses with composite samples.

Loss of elements from solution, nitrogen fractions and ascorbic acid in the leaf, and burn of cured leaf were determined for deficiencies of Fe, Zn, Cu, Mn, B and Mo. Nitrogen fractions were estimated by the Kjeldahl method and its modifications; and P, K, Ca, Mg, Fe, Cu, Mn, B, Na and Al spectrochemically. Variations in S, Zn and Mo were not estimated.

It was found that assumed absorption of biologically essential elements from the complete basal solution adjusted for maximum yields with minimal supply of salts in the spring was affected by the plant without change in proportion of solution components ("economical" solution of Hoagland). Assumed absorption of minerals from the solution by the control plants in the spring approximated 93 % or more. Decreased yields, irrespective of micronutrient deficiency type or low light in fall, caused approximately proportionally increased mineral content in plant and leaf. This yield-supply factor was important with relation to mineral and organic content of the plant; and served as an aid in distinguishing between specific and general effects of micronutrient deficiency. Micronutrient deficiency affected essential element proportions in the leaf to a greater extent than in the plant as a whole.

Micronutrient deficiency, except copper, led to an increased content in the leaf of nitrate and free amino acids and a decrease in protein. Diminished growth caused by deficiency led to decreased production of ascorbic acid in the leaves. Boron and Mo deficiencies were especially effective despite comparatively high yields. Duration of glow when ignited without flame ("burn") of air-dried ("cured") leaf was increased by B and Mn deficiencies.

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SILICON METABOLISM IN DIATOMS. II. SOURCES OF SILICON FOR GROWTH OF NAVICULA PELLICULOSA [1,2,3]

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Richter (22), Bachrach (2), and Pringsheim (21) have reported that small amounts of agar tend to stimulate growth of diatoms in liquid cultures, and both Richter and Bachrach attributed this effect at least in part to the presence of silicon compounds. Likewise Brieger (4) observed in commercial agar the presence of insoluble siliceous matter, including diatom frustules, which might slowly dissolve to furnish sufficient silicon for growth of diatom cells. Patrick and Wallace (20) found that agar, pyridine extract of agar, and ashed extract contained a factor stimulatory to the growth of *Nitzschia linearis*. They concluded that some trace element in the agar was responsible for stimulation, but they did not suggest the possibility that this factor might be silicon.

Apparently there has been no published account of quantitative studies on the availability to diatoms of the soluble silicon in agar and agar ash. This paper deals with evidence indicating that silicon is the factor in agar ash stimulatory to the growth of *Navicula pelliculosa*, and with experimental investigations on the ability of this diatom to utilize various silicon compounds for growth.

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[2] Submitted as National Research Council No. 511-527.
[3] Some of the material included in this paper was presented as part of a Ph.D. thesis submitted to Yale University in 1952.
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Materials and Methods

The diatom used throughout these studies was originally isolated from fresh water in 1949 and has been maintained as a bacteria-free clone on agar slants. It has proved to be a suitable organism for studies of diatom physiology (17, 18).

The basic medium, to which agar, agar ash, or various silicon compounds were added, contained the following concentrations (gm/l) of salts dissolved in distilled water: 0.2 K_2HPO_4, 0.2 $MgSO_4 \cdot 7\,H_2O$, 1.0 $Ca(NO_3)_2 \cdot 4\,H_2O$. Trace elements were added as follows: 0.1 ppm each B, Co, Cu, Mn, Mo; 0.3 ppm Zn; 0.5 ppm Fe. Unless otherwise stated, silicon was added to culture media in the form of orthosilicate, (Fisher, table II). All media were adjusted to pH 7.0 to 7.5 with HCl or KOH. Sterilization was carried out by autoclaving at 15 lbs pressure for 15 minutes.

Special precautions to exclude contamination by silicon leached from glass surfaces were unnecessary. Throughout the experiments described below, controls were run routinely, and at no time was there any evidence that appreciable quantities of silicon from the Pyrex glassware were contaminating the media sufficiently to affect the growth of diatoms. In the absence of added silicon compounds, growth was negligible.

For each concentration of agar, agar ash, or silicon compound tested, 3 or 4 replicates were set up in

125-ml Pyrex Erlenmeyer flasks containing 25 ml of liquid medium. Flasks were inoculated with one loopful from a homogeneous suspension of diatom cells, and shaken constantly at 60 oscillations per minute on a glass-bottomed tray. An illumination of 300 fc was provided by four parallel "White" fluorescent lamps below the tray, and the position of the flasks was changed daily to insure a uniform average illumination. The cultures were maintained at a constant temperature of 23° C.

Throughout these experiments growth of diatoms was virtually uniform in replicate flasks. When growth appeared, one flask of each series was removed from the shaker, the volume of liquid made up to 25 ml to replace that lost by evaporation, 4 drops of 10 % HCl added to dissolve any precipitate of $Ca_3(PO_4)_2$, and the light absorption measured with a Klett-Summerson photoelectric colorimeter, fitted with a ruby glass filter No. 66. Before densities were determined, clumpy suspensions were homogenized by a double passage in a Potter-Elvehjem homogenizer with a Teflon pestle. The sister flasks of the series were treated similarly after intervals of a few days. In those flasks which contained agar or agar ash, the final Klett readings were corrected by subtracting the Klett values given by the medium prior to inoculation. Growth curves were obtained by plotting Klett units against time. A Klett reading of 100 is equivalent to 1.2×10^{10} cells/l.

Silicon (as orthosilicic acid) was determined colorimetrically by a modification of the method proposed by Harrison and Storr (12). Four tenths ml of 5 % ammonium molybdate in 1 *N* H_2SO_4 was added to 4 ml of the silicate solution to form the yellow silicomolybdate complex, and 2 ml of 6 *N* H_2SO_4 were added to eliminate interference from phosphate. Reduction to molybdenum blue was effected by adding 0.4 ml of 5 % aqueous hydroquinone. Each sample was finally made up to 10 ml with distilled water, allowed to stand for 20 minutes, and the color intensity then measured photocolorimetrically. Sodium silicofluoride (Na_2SiF_6), which hydrolyzes in water to give silicic acid (15), served to provide a standard solution containing 2.34 mg Si/l.

Agar ash was prepared by incinerating samples of Bacto-Difco agar in platinum crucibles at 700° to 900° C for two hours. The ash was then added directly to H_2O or mineral medium for silicon determinations and to mineral medium for growth experiments. To remove silicon from the ash, HF was added to the sample and the mixture was heated in a platinum crucible, with additions of H_2SO_4 to convert fluorides to sulfates.

Results

Growth on Various Concentrations of Silicon: Silicon, as potassium orthosilicate, was added in a range of concentrations to both distilled water and mineral medium. Quantitative determinations of the soluble silicon were made both before and after autoclaving the solutions. When silicate was added to mineral medium approximately 17 % disappeared from solution and no longer responded to the molybdate test. On the other hand, the concentration of soluble silicon, both in water and in mineral medium, increased by about 40 % after autoclaving. *N. pelliculosa* was then grown in media containing silicon at various concentrations (fig 1). Growth was proportional to silicon content at low concentrations; 35 mg Si/l gave the best growth and 70 mg Si/l was appreciably inhibitory.

The Effect of Agar on Growth: If 0.1 % agar was added to a mineral medium containing no other source of silicon, some growth of the diatoms occurred (fig 2). Determinations of soluble silicon made on autoclaved solutions of agar in water and in mineral medium showed that 0.1 % agar added 1.5 mg Si/l to the solution. However, agar was stimulatory to growth even in the presence of adequate amounts of silicon (fig 2). It was observed that the cell suspension in the media supplemented with agar was evenly dispersed, whereas that in the media without agar tended to be clumpy. Possibly the increased viscosity of the agar medium facilitated the dispersal of the cells, and thereby promoted growth in some way.

The Effect of Agar Ash on Growth: Agar ash, added to a mineral medium containing no other source of silicon, was found to contain an inorganic factor essential for growth (fig 3). This concentration of ash (equivalent to 1.0 % agar) was found to supply 24 mg soluble Si/l to the mineral medium. The diatom growth in agar ash media was comparable to that in media containing orthosilicic acid of equivalent silicon concentrations.

After treatment with HF to remove all silicon as the volatile SiF_4, agar ash would no longer support growth. It could be shown that this was not a toxic effect caused by HF treatment, since excellent growth took place when the HF-treated-ash media were supplemented with 35 mg Si/l as orthosilicic acid (fig 3). Thus, after removal of silicon from agar ash, there was no evidence for any other inorganic factor stimulatory to growth of *N. pelliculosa*. The addition of vitamins to the medium supplemented with agar ash produced no additional stimulation of growth.

Orthosilicate as a Source of Silicon: In the colorimetric determination of silicon, ammonium molybdate reacts with orthosilicate (7) but not with colloidal silica (9, 11). By comparing the relative growth of *N. pelliculosa* in media containing various silicon preparations with the titres obtained by the molybdate test, it should be possible to determine whether the silicon taken up by diatoms for the formation of their frustules is in the form of orthosilicic acid.

Growth of N. pelliculosa on alkali-treated colloidal silica: Untreated colloidal silica ("Ludox," du Pont Inc.) does not react to give a color with ammonium molybdate, nor does it constitute a source of available silicon for growth of *N. pelliculosa*. As the alkali : silica ratio in a solution increases, the colloidal SiO_2 particles become smaller, until dispersion into SiO_4 tetrahedra is complete (5). It was therefore of in-

terest to test alkali-treated Ludox both in the molybdate reaction and for diatom growth.

The sample of Ludox used in these studies contained 38.64 % SiO_2 or 18.03 % Si. Aliquots of 1.0 ml Ludox, theoretically containing 180 mg total Si, were treated at 25° C for 3 days with KOH in various molar ratios as shown in table I. Each sample was then made up to 10 ml with distilled water, and the soluble silicon was determined with ammonium molybdate. This has been expressed as a percentage of total silicon in table I. It can be seen that only after Ludox has been treated with alkali did it give a color test with ammonium molybdate, indicating the presence of silicon in a soluble form.

Aliquots of each sample were added to mineral nutrient medium to give total silicon concentrations of

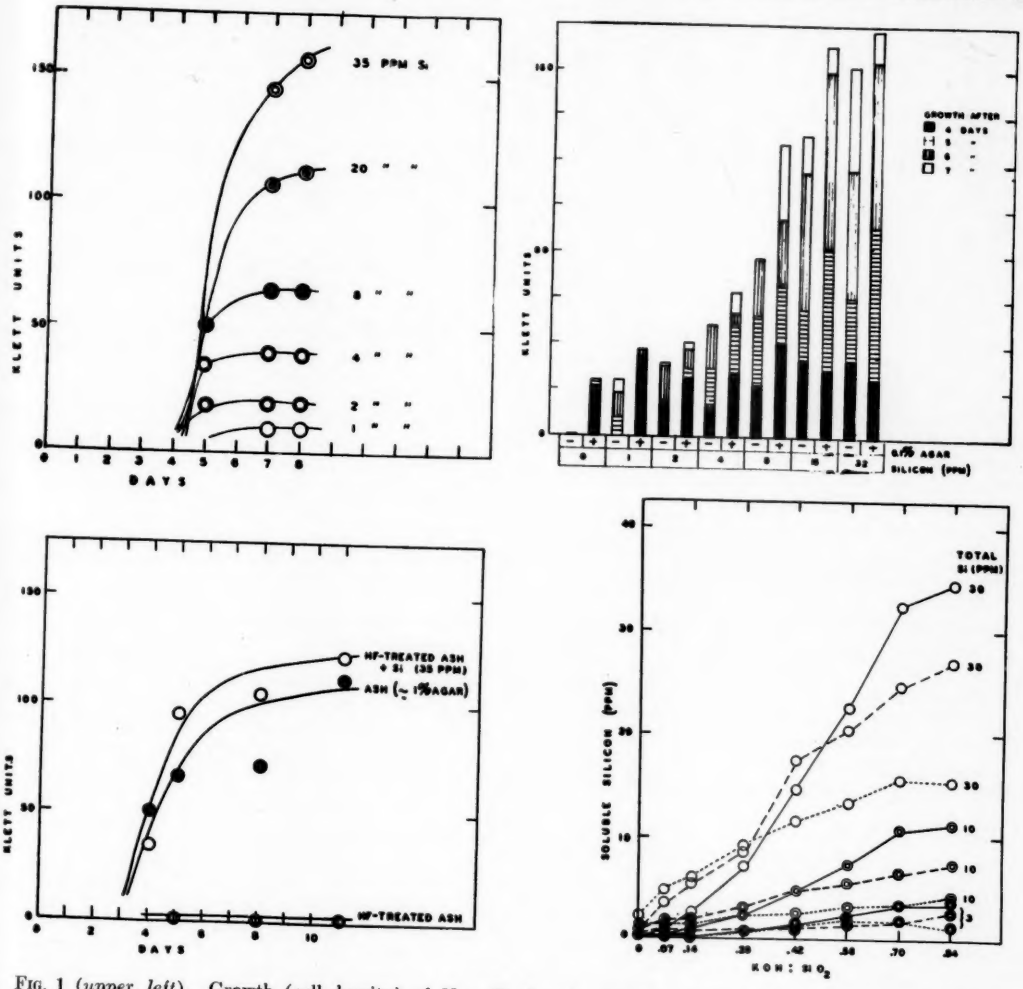

FIG. 1 (*upper, left*). Growth (cell density) of *N. pelliculosa* in media supplemented with orthosilicate. (The Si conc shown is that determined in mineral media after autoclaving.)

FIG. 2 (*upper, right*). Growth (cell density) of *N. pelliculosa* in media supplemented with orthosilicate and in the presence and absence of agar. (The Si conc shown is that determined in mineral media before autoclaving.)

FIG. 3 (*lower, left*). Growth (cell density) of *N. pelliculosa* in media supplemented with the ash of agar and HF-treated ash with and without added silicon.

FIG. 4 (*lower, right*). Soluble silicon liberated upon treatment of Ludox with various amounts of alkali. KOH : SiO_2 represents molar ratios.

Silicon determinations made on samples added to water ————

Silicon determinations made on samples added to mineral medium before autoclaving — — — —

Silicon determinations made on samples added to mineral medium after autoclaving - - - - - - - -

TABLE I

ORTHOSILICATE CONTENT OF MINERAL MEDIA AFTER ADDITION OF ALKALI-TREATED LUDOX SOLUTIONS

KOH : SiO_2 (MOLAR RATIO) EMPLOYED	% SOLUBLE Si IN AQUEOUS SOLN	TOTAL Si CONC (MG/L)					
		3.3		10.1		30.4	
		A*	B	A	B	A	B
0.00	0	0.5	0.5	1.2	1.2	0.6	2.4
0.07	3	0.8	0.5	1.9	1.2	3.6	4.8
0.14	8	..	0.6	2.1	1.5	5.4	6.0
0.28	23	0.9	0.8	3.2	2.3	8.6	9.1
0.42	48	1.3	0.9	4.7	2.5	16.9	11.5
0.56	74	1.6	2.0	5.5	3.2	20.4	13.3
0.70	110	1.8	1.9	6.5	3.6	24.7	15.5
0.84	112	2.5	1.3	7.3	4.2	27.0	15.3

* Amount (mg/l) detected after addition to mineral media. A—before autoclaving. B—after autoclaving.

3.3, 10.1, and 30.4 mg/l. The amount of silicon in true solution depended on the extent to which colloidal SiO_2 had been broken down to silicate and on the quantity of Ca silicate precipitated. The amounts of soluble silicon, based on molybdate determination, present in each solution before and after autoclaving, are shown in table I. The orthosilicate concentrations and growth of diatoms in media prepared using such treated Ludox samples are illustrated graphically in figure 4 and figure 5, respectively.

From these data it can be concluded that the more extensively the colloidal silica has been hydrolyzed by alkali, the greater is the resulting growth.

Growth of N. pelliculosa on other silicon compounds: Other compounds tested in various concentrations as sources of silicon for *N. pelliculosa* are listed in table II. Apparently only those compounds which in solution yield silicon in a form available to react with ammonium molybdate, presumably orthosilicic acid, can serve as sources of silicon for *N. pelliculosa*. The cells are not able to extract silicon from large colloidal particles such as those of Ludox, nor are they able to use a substituted orthosilicic acid in which one hydroxyl group has been replaced by a methyl group.

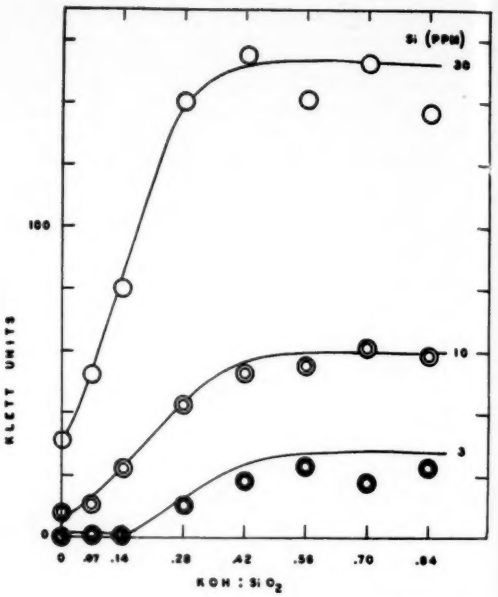

FIG. 5. Growth (cell density) of *N. pelliculosa* after 12 days in media supplemented with alkali-treated Ludox employed to give a total silicon concentration of 3, 10 and 30 mg/l. KOH : SiO_2 represents molar ratios.

DISCUSSION

Virtually all species of diatoms cultured in the laboratory on synthetic media have been reported to exhibit an absolute requirement of silicon for growth. Certainly this is true for all those investigated in this laboratory, in which 60 pure strains, representing at least 6 genera and 15 species, have been tested. Hy-

TABLE II

COMPOUNDS TESTED AS SOURCES OF SILICON FOR NAVICULA PELLICULOSA

COMPOUND	CONC	SOLUBLE Si	GROWTH AFTER 10 DAYS
	mg dry wt/l	*mg/l*	*Klett units*
Orthosilicate, $K_2O : SiO_2 = 2 : 1$ (Fisher, 423990/F33)	200	20	150
Mixture of $K_2Si_2O_5$ and $K_2Si_4O_7$ (Fisher "K_2SiO_3," C.P., P-298/510740)	200	1.4	10
"Metasilicate," $K_2O : SiO_2 = 1 : 1.5$ (Fisher)	200	18	99
Potassium silicate, $K_2O : SiO_2 = 1 : 3.3$ (Sylvania, PS-4, TT-Q-3) 32.7 % SiO_2 found	60	11	93
Ludox colloidal silica, $SiO_2 : Na_2O = 285 : 1$, 29.2 % SiO_2; found 32.7 % SiO_2 (du Pont) 38.6 % SiO_2 found	260, 26	0	0
Sodium methyl siliconate, $CH_3SiO_{1.5}$—20 %, Na_2O—9.5 % (Canadian Gen. Elec. Co., SC-50)	200, 70, 20	0	0
Sodium methyl siliconate powder, $CH_3SiO_{1.5}$—60 %, Na_2O—25.5 % (Canadian Gen. Elec. Co., 81362)	200, 70, 20	0	0

drated silica exists in a number of different forms, from large colloidal particles to the simpler dissolved silicic acid and orthosilicate ions. Although a few investigators have tested various sources of silicon for their availability to diatoms (2, 8), no attempt has been made to determine more precisely the form of silicon utilized for diatom growth. This is in part attributable to the fact that pure silicon compounds of known chemical composition are not readily available. Some of the published results are summarized in table III. It will be seen that there are many discrepancies, some of which can be explained by the various concepts of "good" growth in terms of the rate of cell division and final cell density.

At the pH of media employed in these investigations the dissolved silicon would be predominantly in the form of orthosilicic acid, or hydrated SiO_2, some of which might be slightly aggregated. The equilibrium would be shifted toward the monomer as the orthosilicate is removed from solution by diatoms. The large colloidal particles of Ludox are free neither to react with ammonium molybdate nor to be utilized for diatom growth. However, untreated Ludox depolymerized slightly in the alkaline medium (fig 4, 5) and this breakdown might be expected to continue slowly at room temperatures until finally diatom growth could occur even in media supplemented with untreated Ludox.

The amount of silicate in the medium is a vital factor controlling the density of a diatom culture when other nutrilites are present in sufficient quantities (14). Although silicon in natural waters is present at concentrations ranging from about 0.1 to 3.0 mg Si/l, it was found that very dense cultures of *N. pelliculosa*, containing approximately 10^{10} cells/l, could only be obtained when the concentration of silicon added to the medium was of the order of 15 to 30 mg/l. In most recent studies of diatom physiology, it would appear that adequate amounts of silicon have been incorporated in the nutrient media, although some of these may not have been sufficient for maximum growth. Examples of the concentrations, given as mg Si/l, employed by various workers are: 4.7 for *Navicula distans* (16), 23 for *Navicula minima atomoides* (25), 10, 14, 23 for *Nitzschia palea* (6, 13, 28), 4.5 for *Nitzschia linearis* (20), and 12 for *Asterionella formosa* (19).

Bacto-Difco agar is stated to contain 0.9 % SiO_2, equivalent to 4.2 mg silicon per gram. In the original material the silica may be present largely in the form of frustules of epiphytic and other associated diatoms. The process of incineration leaves a quantity of free alkali in the ash, which aids in the solution of these frustules. Virtually all of the silica in agar is thereby liberated in a soluble form.

Although it has been demonstrated that the stimulatory action of agar ash is to be attributed to its content of silicon in a soluble form, some residual effect in the case of agar itself (0.1 %) remains to be explained. Though agar has been shown to contain small quantities of vitamins (23, 24), it has not been possible to demonstrate any stimulating effect of vitamin mixtures on the growth of *N. pelliculosa*. Some authors (10, 28) have suggested that particles of such substances as agar or glass wool may promote diatom growth in liquid culture by providing a solid substratum, but it is difficult to understand how this would be beneficial. Agar may also act as a buffer (26) and this property may contribute to its favorable action when added to such weakly buffered media as those customarily employed for the cultivation of

TABLE III

GROWTH STUDIES OF DIATOMS

SPECIES	SOURCE OF Si	GROWTH	REF
Nitzschia palea	$CaSi_2O_5$	Best	(22)
	$K_2Si_2O_5$	Good	
	$Na_2Si_2O_5$	Good	
	Glass walls of culture flasks only	Some	
Coscinodiscus excentricus	Glass walls of culture flasks only	Some	(1)
	K silicate fragments	Some	
	Precipitated Ca silicate	Some	
	Pure dialyzed silica	Some	
Nitzschia linearis	Washed kaolin	Excellent	(8)
	Powdered orthoclase feldspar	Excellent	
	Pure pulverized potter's clay	Excellent	
	Vitreous silica	None	
	Silica gel	None	
	Powdered glass	None	
	K or Na silicate	None (possibly conc. or pH inhibitory), (3))	
Fragilaria elliptica *Navicula* sp. *Nitzschia palea* *Rhopalodia gibba* *Melosira varians* *Nitzschia palea* *Nitzschia sigmoidea*	$K_2Si_2O_5$	Good	(4)
Navicula sp.	Silica gel	Good (no pit formation)	
Fragilaria elliptica	Silica gel	Good (pit formation noted)	
Nitzschia palea	Glass walls of culture flasks only	Poor	(2)
	Agar	Excellent	
	Precipitated SiO_2	Good	
	Powdered quartz	Some	
	Powdered opal	Some	
Nitzschia palea *Navicula minuscula*	Nacrite crystals	Good, dissolution of crystals noted	(27)
Nitzschia palea	Na_2SiO_3 (impure)	None	(13)
	Na_2SiO_3 (pure)	Excellent	

algae. Finally, the favorable effect of small quantities of agar on the growth of diatoms may be attributable in part to its action as a dispersing agent. Wetter (29) has found that 0.1 % agar promotes growth of *Aspergillus niger* owing to better nutrient conditions for the conidia resulting from their homogeneous dispersion in a medium of higher viscosity.

Summary

Navicula pelliculosa (Bréb.) Hilse has an obligate requirement for silicon. Growth is proportional to soluble silicon at low concentrations, and is most dense in media containing about 35 mg Si/l. Since the addition of 1.0 % agar supplies 1.5 mg soluble Si/l to the nutrient medium, such media can support some diatom growth in the absence of any other source of silicon. The ash of agar, employed at a concentration equivalent to 0.1 % agar, supplies 24 mg soluble Si/l and thus supports good diatom growth. By treatment with HF this silicon can be removed from agar ash, which thereby loses its essential growth factor. When 0.1 % agar is added in the presence of adequate amounts of silicon, there is some acceleration of growth, possibly attributable to its action as a dispersing agent.

Orthosilicates, which react with ammonium molybdate, serve as suitable sources of silicon for growth of *N. pelliculosa*. Colloidal silica (Ludox) neither reacts with ammonium molybdate nor supports diatom growth unless it has first been depolymerized with alkali.

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ROLE OF MOLYBDENUM AS A CONSTITUENT OF NITRATE REDUCTASE FROM SOYBEAN LEAVES[1,2]

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It has already been shown that the nitrate reductase of soybean leaves which catalyzes the reduction of nitrate to nitrite by reduced diphospho- or triphosphopyridine nucleotide (DPNH or TPNH) is a metallo-flavoprotein with flavin adenine dinucleotide (FAD) as the prosthetic group (1). The metal component of a similar, but TPNH-specific, nitrate reductase from Neurospora (2) has recently been identified as molybdenum (3, 4). Molybdate as well as FAD or the mononucleotide (FMN) were shown to function as electron carriers in the enzymatic transfer of electrons from TPNH to nitrate to yield nitrite (5, 6).

The purpose of this paper is to present similar evidence identifying molybdenum as the metal constituent of nitrate reductase from soybean leaves, and to show that during the enzymatic transfer of electrons from DPNH to nitrate, both flavin and molybdenum function as carriers in the following sequence:

$$\text{TPNH} \rightarrow \text{FAD (or FMN)} \rightarrow \text{Mo} \rightarrow NO_3^-$$

MATERIALS AND METHODS

The crude enzyme extract was obtained from the leaves of 10-day to 5-week-old soybean plants grown in flats containing vermiculite and supplied daily with a complete nutrient solution as previously described (1). The enzyme extract was prepared essentially as described (1) except that the alumina powder was omitted and cysteine and versene were added to the phosphate buffer, each at final concentrations of 10^{-4} M. The clear green supernatant solution resulting from high speed centrifugation was fractionated with calcium phosphate gel and ammonium sulfate in the usual way (1); and the second calcium phosphate gel adsorption eluate was used for most of these studies.

The preparation of the various cofactors, substrates and other materials used in this investigation has already been indicated (1). Reduced flavin ($FADH_2$ or $FMNH_2$) was prepared (6) in Thunberg tubes with hydrosulfite followed by hydrogen gas bubbling and evacuation.

Molybdenum-free nitrate reductase was prepared by successive dialysis against cyanide- and molybdenum-free phosphate-glutathione solutions as described elsewhere (4) except that shorter dialysis times were used. In view of the almost complete loss of activity upon dialysis for 6 or more hours, the soybean enzyme was dialyzed against 10^{-3} M cyanide solution and subsequently molybdenum-free phosphate and glutathione solution for 3 and 2 hours, respectively.

For molybdenum assay, cell-free extracts and the various protein fractions were digested with distilled HNO_3; and molybdenum was determined in suitable aliquots of the cooled and diluted digests by the *Aspergillus niger* assay (4).

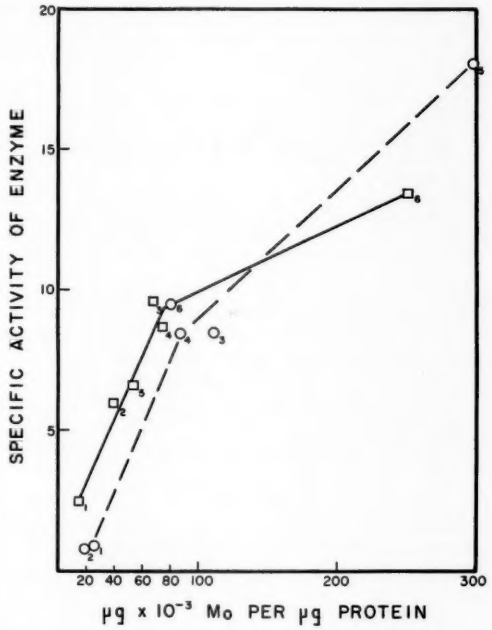

FIG. 1. Relation between molybdenum content and specific activity of nitrate reductase. Circles (○) and squares (□) represent the various protein fractions of two independent fractionations. Subscript 1 is the crude extract, $\bigcirc_2$ is the 0 to 40% ammonium sulfate precipitate of the supernatant solution after the first calcium phosphate gel treatment; $\square_2$, $\square_3$ are the first and second eluates, respectively, of the first calcium phosphate gel; $\bigcirc_3$ is the eluate of the first calcium phosphate gel; subscript 4 is the 0 to 40% ammonium sulfate supernatant solution of the first calcium phosphate gel eluate; subscript 5 is the 0 to 40% ammonium sulfate precipitate of the first calcium phosphate gel eluate; subscript 6 is the eluate of the second calcium phosphate gel.

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[2] Contribution No. 101 of the McCollum-Pratt Institute. This investigation was supported in part by research grants (G-2332) from the National Institutes of Health, U. S. Public Health Service, and from the Climax Molybdenum Company, New York City, New York.

[3] McCollum-Pratt Institute Postdoctoral Fellow on leave from the University of Bristol, England.

TABLE I

EFFECT OF METALS ON REACTIVATION OF CYANIDE-DIALYZED NITRATE REDUCTASE

TREATMENT	UNITS OF ENZYME ACTIVITY/0.2 ML ENZYME	
	EXPERIMENT I	EXPERIMENT II
I—Undialyzed enzyme ...	7.3	7.1
II—Single dialysis *	6.0	5.2
III—II + cyanide (10^{-3} M)	0	1.6
IV—Successive dialysis **	0	1.0
Added metals		
MoO_3	...	6.2
Na_2MoO_4	...	5.8
$FeCl_3$	...	1.2
$FeSO_4$	...	1.3
$ZnSO_4$	...	1.0
$MnSO_4$	...	1.0
$CoSO_4$	...	1.3
$NiCl_2$	...	1.2
AgCl	...	1.0
$NaWO_4$	...	1.5
$Na_2Cr_2O_7$	...	1.1
Na_3VO_4	...	1.5
$VO_2(NO_3)_2$	...	1.5
$Na_2B_2O_4$	...	1.0

Enzyme assay as described under "Materials and Methods."

* Dialyzed for 3 hrs against 0.1 M phosphate + 10^{-3} M glutathione. All reagents were freed from Mo as indicated (4).

** As for treatment III followed by redialysis for 2 hrs as in treatment II. 0.2 ml enzyme contained 107 μg protein. 1 μg of each metal added to 0.2 ml of enzyme and incubated for 10 min at room temperature before enzyme assay.

Enzyme activity was measured by the colorimetric determination for nitrite as detailed earlier (1). A period of 20 minutes, unless otherwise stated, was generally used for the enzymatic reaction time. Specific activity is expressed as units or 10^{-9} moles of nitrite formed per mg protein. Protein was determined by the Folin method of Lowry et al (7).

RESULTS

Figure 1 shows that there is a direct relationship between specific activity and the molybdenum content per mg protein of the various enzyme fractions. The data represent the results of two independent experiments. The highest activity occurs in the second calcium phosphate gel eluate which also has the greatest molybdenum content. A semiquantitative colorimetric determination for Zn, Cu, Pb, Ni, Co, Hg, Cd, Ga, and Bi, by extraction with dithizone failed to show any consistent relationship between these metals collectively and specific activity.

As shown in table I, a partially purified fraction of nitrate reductase was freed of its metal component by successive dialyses against cyanide- and molybdenum-free phosphate-glutathione solutions for 3 and 2 hours, respectively. The addition of molybdenum trioxide or sodium molybdate reactivated the enzyme to 85 % of the original value. Preincubation in place of molybdenum with other trace metals including Fe^{+++} and Fe^{++}, Zn, Mn, Co, Ni, Ag, W, chromate, V and B, respectively, did not restore the activity of nitrate reductase (table I).

TABLE II

$FMNH_2$ AS AN ELECTRON DONOR FOR THE ENZYMATIC REDUCTION OF NITRATE

REACTION MIXTURES	DPNH FMN KNO_3 ENZYME	KNO_3 – DPNH + $FMNH_2$* ENZYME	KNO_3 – DPNH + $FMNH_2$ MO-FREE ENZYME**	KNO_3 – DPNH + $FMNH_2$ BOILED ENZYME†
mμM NO_2^- formed ..	9.5	8.7	2.0	1.6

Reaction mixtures contained as indicated: 0.3 ml enzyme containing 156 μg protein, 0.24 ml of DPNH (2 μM per ml), 0.24 ml of FMN (0.09 μM/ml), 0.1 ml of 0.1 M KNO_3, and 0.1 M pyrophosphate buffer, pH 7.0 to give a final volume of 3.0 ml. The reactions were started by tipping the other reactants into the reduced flavin in pyrophosphate buffer in a Thunberg tube anaerobically. 0.5 ml of reaction mixture tested for nitrite with sulfanilamide and 1-naphthyl ethylenediamine dihydrochloride.

* Reduced flavin was prepared by reduction with $Na_2S_2O_4$ and H_2 as described (6).

** Mo-free enzyme dialyzed successively in phosphate glutathione cyanide solution as described elsewhere (4).

† Boiled enzyme prepared by heating at 100° C for 10 min was substituted for native enzyme.

TABLE III

DITHIONITE-TREATED MO AS AN ELECTRON DONOR FOR THE ENZYMATIC REDUCTION OF NITRATE

REACTION MIXTURES	DPNH FMN KNO_3 ENZYME	REDUCED MO* – DPNH	REDUCED MO – DPNH – FLAVIN	MO – DPNH	REDUCED MO – DPNH MO-FREE ENZYME	REDUCED MO – DPNH BOILED** ENZYME
mμM NO_2^- formed	9.5	9.0	9.3	2.0	1.6	1.4

Reaction mixtures contained as indicated: 0.3 ml enzyme containing 205 μg protein, 0.24 ml of DPNH (2 μM per ml), 0.24 ml of FMN (0.09 μM/ml), 0.1 ml of 0.1 M KNO_3, and 0.1 M pyrophosphate buffer pH 7 to give a final volume of 3.0 ml. The reactions were started by tipping the other reagents into the molybdate in phosphate buffer in a Thunberg tube anaerobically. 0.5 ml of reaction mixture tested for nitrite with sulfanilamide and 1-naphthyl ethylenediamine dihydrochloride.

* Reduced molybdate was prepared in Thunberg tubes as indicated (5).

** Boiled enzyme prepared by heating at 100° C for 10 min was substituted for native enzyme.

The results in table II show that reduced flavin can replace TPNH as an electron donor for the enzymatic reduction of nitrate to nitrite. The molybdenum component of the enzyme is necessary for the above reduction as shown by the failure of the molybdenum-free nitrate reductase to mediate the reaction.

Table III indicates that reduced molybdate prepared with $Na_2S_2O_4$ and H_2 can serve anaerobically as an electron donor in the absence of added DPNH and flavin for the enzymatic conversion of nitrate to nitrite. The addition of flavin does not increase the enzymatic rate of nitrate reduction by reduced molybdate.

Discussion

Molybdenum has already been identified as the metal component of Neurospora nitrate reductase (3, 4) and its specific role as an electron carrier elucidated (5, 6). The large volume of evidence pointing to a function of molybdate in nitrate assimilation by higher plants (8) as well as the similarity of nitrate reductase of soybean leaves with that of Neurospora prompted the present investigation concerning the identify of the metal component of the higher plant enzyme. The preceding experiments showing the direct proportionality between molybdenum content and specific activity as well as the specific reactivation of the dialyzed enzyme by molybdenum have identified molybdenum as the constituent of nitrate reductase in the leaves of the soybean plant.

The experiments with reduced flavin and dithionite-treated molybdate, modeled after the more detailed and elaborate experiments with Neurospora (6) (tables II and III), have indicated that the pathway of electron transport in the enzymatic reduction of nitrate by DPNH proceeds through flavin and molybdenum in much the same manner as has been established for Neurospora, namely:

$$\text{DPNH} \rightarrow \text{FAD (or FMN)} \rightarrow \text{Mo} \rightarrow NO_3^-$$

The questions of whether one or more proteins are involved in the above series of reactions is not settled (6). If the enzyme proves to be a single protein, and thus far it has not been possible to demonstrate otherwise by fractionation techiques, it would represent a versatile system in view of its ability to catalyze each of the above reaction steps, very likely at different sites on the enzyme molecule. Other metalloflavin systems such as butyryl-coenzyme A dehydrogenase, DPN-cytochrome c reductase, xanthine oxidase and aldehyde oxidase are presumably single enzymes which catalyze and stepwise transfer of electrons by way of a number of carriers. The latter two systems are molybdoflavoproteins which can be reactivated after removal of molybdenum by molybdenum trioxide and not by molybdate (9, 10). Latimer and Hildebrand (11) state that molybdenum may form compounds having positive oxidation states of 2, 3, 4, 5 and 6. The fact that either $MoO_4^=$ or MoO_3 (both of oxidation state + 6) reactivates the purified nitrate reductase suggests this form as the more oxidized state of the metal in the enzyme. The identity of the less positive oxidation state which the metal assumes during electron transport is not known. By analogy with cytochrome c reductase, polyphenol oxidase, and ascorbic acid oxidase where the iron and copper undergo single electron changes one might speculate that molybdenum is reduced to the + 5 oxidation state.

It may be of interest that the nitrate reductase system does not show an inorganic phosphate requirement in contrast to that reported for xanthine and aldehyde oxidases (9, 10); and that the latter two systems also appear to contain an iron component (10, 12).

Summary

Molybdenum, already established as the metal component of Neurospora nitrate reductase, has also been identified as the metal of nitrate reductase of soybean leaves. This is supported by the proportionality between molybdenum content and specific enzyme activity, as well as the restoration of dialyzed enzyme specifically by MoO_3, or Na_2MoO_4. Other micronutrient elements including Fe, Cu, Mn, Co, Ni, W, V and B, respectively, were without effect. It has also been shown that during the enzymatic transfer of electrons from DPNH to nitrate, flavin and molybdenum function as electron carriers in the following sequence:

$$\text{DPNH} \rightarrow \text{FAD (or FMN)} \rightarrow \text{Mo} \rightarrow NO_3^-$$

$FMNH_2$ or reduced molybdate (without added flavin) can replace reduced pyridine nucleotides in the enzymatic reduction of nitrate to nitrite.

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PURIFICATION OF CHLOROPLASTS BY A DENSITY TECHNIQUE[1,2,3]

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Since the classical experiments of Granick (6) it has been possible to isolate and purify chloroplasts by differential centrifugation. Recently, it became evident (7) that differential centrifugation is inadequate to separate young small chloroplasts from the small non-plastid particles inferred to be present in leaf homogenates (10). It therefore appeared desirable to develop alternate methods of purifying chloroplasts.

Differential centrifugation separates particles according to size, shape and density, but primarily according to size. Young chloroplasts are closer in size to mitochondria than are mature chloroplasts, and this is the main obstacle to their separation. Another possibility appeared to be the use of the particle densities alone to effect separation. Accordingly, preparations of mixed mature chloroplasts and mitochondria were suspended in 80 to 100 % glycerol solutions in water, and then centrifuged vigorously in the Spinco ultracentrifuge. In this procedure most of the green material, and therefore most of the chloroplasts, were found to float to the top of the tube. Further experiments showed that the viscosity of these solutions could be reduced by using 50 % glycerol by volume and adding sufficient sucrose to make the final preparation 0.4 M in sucrose.

The experiments reported here will deal with the question of the degree of purification achieved with this flotation technique, and its applicability to young chloroplasts.

MATERIALS AND METHODS

The sources of leaf tissues were spinach purchased at a local grocery, tobacco leaves from Turkish tobacco (variety Samsun) grown in six-inch pots in the greenhouse, and primary leaves of red kidney bean plants grown in soil in flats in the greenhouse.

In making preparations of particles, leaves were ground in an Omnimixer at 50 to 60 % of line voltage, in four times their weight M/15 pH 7.0 phosphate buffer made to 0.4 M sucrose. The resulting homogenate was strained through a pad of cheesecloth and glass wool. Centrifugation of this homogenate at 1,000 × g for 12 minutes yielded a pellet containing chloroplasts, some mitochondria, and remains of nuclei. This pellet was resuspended in phosphate-sucrose buffer, and constituted the "crude particle" preparation, in most cases.

For centrifugation in a dense medium, a 2-ml aliquot of the original particle preparation was placed in a 13-ml Spinco centrifuge tube, together with 5.0 ml glycerol and 3.0 ml of a concentrated sucrose solution containing 1.30 gm of sucrose, and the contents of the tube were mixed thoroughly. The fluid in the tube then had a density of 1.17 gm/ml. When the

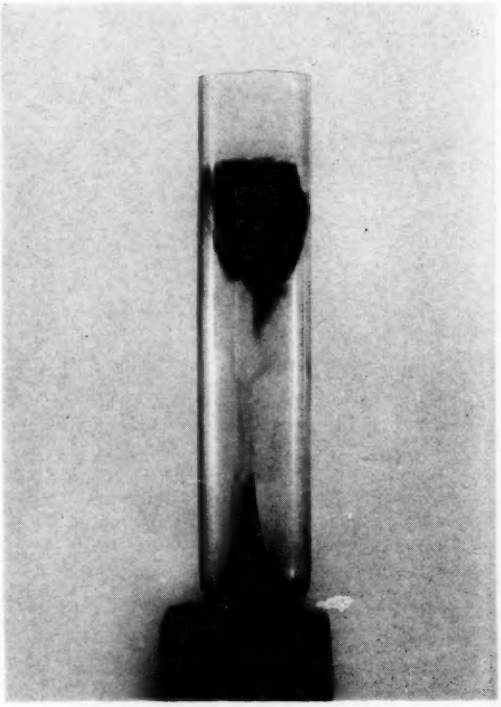

FIG. 1. Centrifuge tube after removal of fluid. Notice the pellet adhering to wall of tube at top, and bottom pellet at the opposite side of the tube.

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[3] This work was supported in part by grant No. C-2180 C, M and G from the National Cancer Institute, National Institutes of Health, Public Health Service.

density was to be varied, the amount of glycerol was changed, and the volume of the original particle preparation was adjusted to keep the total volume equal to 10.0 ml in all cases.

After centrifugation at 40,000 rpm in the Spinco No. 40 rotor, a very distinct, compacted pellet of floating material was observed at the top of the tube. In the middle, the glycerol-sucrose solution was a pale green color; and a mixed green and white pellet was observed at the bottom of the tube (see fig 1). The top pellet, middle fluid and bottom pellets were separated in most cases. Top and bottom pellets were resuspended in sucrose-buffer or plain phosphate buffer, depending on the tests to be performed. The fluid was diluted with glycerol-free buffer and centrifuged at 10,000 × g for 20 minutes. The particles thus collected from the fluid were resuspended in the same medium as the top and bottom pellets.

The time for flotation and sedimentation to achieve completion was determined by measuring the total amount of chlorophyll remaining in the fluid after various periods of centrifugation (fig 2). It can be seen that sedimentation and/or flotation of chloroplast material is almost complete in 15 minutes, and has gone to completion in 30 minutes. The fluid always contained some chlorophyll, but this was most probably in chloroplastic particles having the same density as the suspending medium. Some chloroplasts were always sedimented, and some always floated, at almost any density over 1.13 gm/ml. This would indicate a range of chloroplast densities in all preparations. Some would, therefore, be expected to have exactly the same density as the medium, and these would neither float to the top nor be sedimented to the bottom.

Protein N content of the preparations was determined by the Nessler procedure after digestion of washed trichloroacetic acid precipitates. Chlorophyll was determined by the modification of Arnon (1). Catalase tests were made according to the procedure developed by Feinstein (5).

Nucleic acid in the preparation was determined, following precipitation of the nucleoproteins with 95% alcohol, according to the method of Ogur and Rosen (9). The final acid extracts, containing hydrolyzed nucleic acid componenets, were analyzed for phosphorus and for ultraviolet absorption. No attempt was made to separate RNA from DNA by preferential extraction with cold perchloric acid; rather, the washed pellets were extracted only with $N/2$ perchloric acid at 70° C for 20 minutes, and total nucleic acid was determined. Values are converted into μgm nucleic acid P/mg protein N. Phosphorus was determined by the method of Berenblum and Chaim (2).

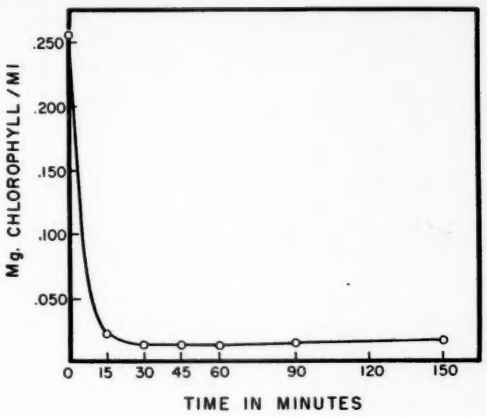

FIG. 2. Chlorophyll remaining in the fluid phase of the Spinco ultracentrifuge tube after varying times of centrifugation. Most of the tobacco chloroplasts have either floated to the top or been sedimented to the bottom after 30 min. Density of the suspending fluid is 1.17 gm/ml; speed of centrifugation is 40,000 rpm in the Spinco No. 40 rotor preparative ultracentrifuge.

Isotopic nitrogen was applied by immersing detached leaves in a 0.01 M solution of 32 atom percent excess $N^{15}H_4Cl$ for 15 minutes, then removing the leaves and permitting them to incubate in the dark. After four hours, the leaves were ground, the various particulate fractions separated according to the described procedures. The NH_3 from digested pellets was distilled into HCl, converted to N_2 with alkaline

TABLE I

CHLOROPHYLL, PROTEIN N AND CATALASE IN SEPARATED SPINACH PARTICLES

FRACTION	CATALASE/ML **	CHLOROPHYLL/ML	PROTEIN N/ML	CATALASE/N	CHLOROPHYLL/N
	millimoles perborate †	*mg*	*mg*	*activity/mg*	*mg/mg*
Crude particles *	0.158	0.170	0.180	0.88	0.95
Bottom pellet	0.124	0.010	0.032	3.90	0.31
Middle fluid	0.010	0.030	0.037	0.37	0.81
Top pellet	0.018	0.112	0.106	0.17	1.06
Recovery	0.152	0.152	0.180	...	...

* Original particles sedimented from spinach leaf homogenate at 1,000 × g for 12 min.
** All measurements of separated fractions calculated back to ml of crude particles.
† Catalase measured as millimoles of perborate destroyed in 5 min by the enzyme.

TABLE II

CHLOROPHYLL, PROTEIN N AND CATALASE IN SEPARATED TOBACCO LEAF PARTICLES

FRACTION	CATALASE/ML *	CHLOROPHYLL/ML	PROTEIN N/ML	CATALASE/N	CHLOROPHYLL/N
	millimoles perborate	*mg*	*mg*	*activity/mg*	*mg/mg*
Crude particles	1.74	1.25	1.18	1.36	0.97
Bottom pellet	0.73	0.20	0.29	2.52	0.69
Middle fluid	0.08	0.32	0.25	0.32	1.28
Top pellet	0.09	0.90	0.69	0.10	1.30
Recovery	0.90	1.42	1.23	...	...

* All measurements of separated fractions calculated back to ml of crude particles.

hypobromite, then isotope content measured in a Consolidated-Nier mass spectrometer.

RESULTS

ANALYSIS OF FLOATING VS SINKING PARTICLES: It has been shown previously (7) that chloroplasts purified by washing are relatively free of catalase activity, and are enriched in chlorophyll per mg N. These criteria were accordingly applied to floating and sedimenting material.

A crude particle preparation from spinach was separated into bottom pellet, middle fluid, and top pellet as described. An aliquot of the original particles was saved for later tests, prior to the separation by density. The results of one experiment are shown in table I; they essentially duplicate many other similar experiments. It can be seen that the bottom and top pellets differ markedly in their chlorophyll and catalase contents. The top pellet has 0.17 units of catalase activity per mg protein N, and the bottom pellet 3.90 units per mg N, or 45 times as much. The reverse obtains for chlorophyll per mg protein N; the top pellet is enriched, containing 1.06 mg chlorophyll per mg N, while the bottom pellet has only 0.31 mg chlorophyll per mg N. A crude particle suspension was intermediate for both of these measurements. It is obvious from this that the suspension of crude particles has been separated into two distinct fractions, the bottom pellet containing most or all of the non-chloroplastic material as well as a few chloroplasts, and the top pellet containing chloroplasts much purified compared to the original suspension.

An identical experiment with tobacco particles is shown in table II. Here the top pellet has two times as much chlorophyll per mg N as the bottom pellet; and the bottom pellet has 25 times the concentration of catalase as the top.

Another criterion for the purity of chloroplasts, as established previously by analysis of washed chloroplasts, is the absence, or, at least, presence in very small amount, of nucleic acid. A number of analyses were made of the nucleic acid content of the fractions under investigation; the results of eight experiments with tobacco are shown in table III. In four cases, washed chloroplasts were prepared from the same homogenates that supplied the crude particles. The picture that emerges quite clearly is that the top pellets uniformly have only 2 to 3 μgm nucleic acid P

TABLE III

TOTAL NUCLEIC ACID CONTENT OF TOBACCO FRACTIONS OF DIFFERENT DENSITY COMPARED TO THAT OF WASHED CHLOROPLASTS

EXPT.	FRACTION				
	CRUDE PARTICLES *	BOTTOM PELLET	FLUID	TOP PELLET	WASHED CHLOROPLASTS
	μgm P/mg N	*μgm P/mg N*	*μgm P/mg N*	*μgm P/mg N* †	*μgm P/mg N* †
1	12.0	13.5	...	3.0	...
2	9.0	19.5	...	2.7	...
3	14.0	54.0	...	2.8	...
4	8.0	18.0	4.0	1.1	...
5	16.0	55.0	...	2.0	5.5
6	8.0	22.0	1.2	2.3	2.9
7	7.2	19.5	2.0	2.5	4.5
8	...	43.0	...	3.3	5.5
9 **	...	30.0	...	25.0	...

* All crude particles except for expt 9, prepared by centrifuging tobacco homogenates at 1,000 × g for 12 min.
** Crude particles of expt 9 were not sedimented by 1,000 × g, but were sedimented at 10,000 × g for 10 min.
† The values reported are maximal, assuming that all absorption at 260 mμ and all the P found is strictly due to nucleic acid. A clean peak was never found in these absorption spectra (with the exception of expt 9).

per mg N, or usually 1/3 to 1/5 the concentration found in the crude particles. In these experiments, the nucleic acid content is even lower than that of the washed chloroplasts.

Nucleic acid content of the crude particle suspensions lies between 7.2 and 16 μgm P per mg N. Part of this variability is due to the fact that in different experiments, varying degrees of attention were paid to removing nuclei before analyzing the crude particles.

Nucleic acid content of the bottom pellets ranges from 13.5 to 55 μgm nucleic acid P per mg N. It should be pointed out, however, that the values shown are nucleic acid per unit total N. There seems to be some variation in the amount of chloroplast material that sedimented from time to time; and the low values for nucleic acid probably occur when there is a large amount of chloroplast material in the bottom pellet.

The outstanding observation, though, is that, in spite of the variability found in the crude particles and in the bottom pellet, the top pellets all contain material that is uniformly very low in nucleic acid.

In addition, the ultraviolet spectra of extracts from these top pellets always showed a large amount of absorption in the 230 to 255 mμ region, so that a distinct peak at 260 mμ was never seen. Chloroplasts purified by repeated centrifugal washings gave nucleic acid extracts with the identical absorption spectrum, i.e., only a shoulder at 260 mμ, not a distinct peak. By contrast, nucleic acid extracts of the crude particle suspension and especially of the bottom pellet, showed relatively clean nucleic acid absorption spectra.

A final criterion applied to the top pellet preparations was that of their ability to incorporate labelled nitrogen from N^{15} ammonia supplied to detached leaves in the dark. The results of this experiment are shown in table IV.

During the 4-hour incubation period, original particles achieved an atom percent excess of 0.355. The bottom pellet separated from these had 0.463 atom percent excess, even though it still consisted of over 50 % chloroplasts, as judged from the chlorophyll/N ratio of 0.75. Calculations indicate the nonchloroplast N of the bottom pellet would have an atom percent excess of 0.96, if free of chloroplasts. Washed chloroplasts had an atom percent excess N^{15} ratio of 0.230, which was almost matched by the top pellet with a ratio of 0.253. The respective chlorophyll/N ratios are 1.06 and 1.10. Thus, a low activity in incorporating N^{15} accompanies a high chlorophyll/N ratio in the top pellet isolated by this procedure, just as it does when chloroplasts are purified by washing.

TABLE IV

INCORPORATION OF ISOTOPIC NITROGEN INTO FRACTIONS OF DIFFERENT DENSITY, COMPARED TO WASHED CHLOROPLASTS

FRACTION	MG CHLOROPHYLL/MG N	ATOM % EXCESS N^{15}
Crude particles	0.96	0.355
Bottom pellet	0.75	0.463
Top pellet	1.10	0.253
Washed chloroplasts	1.06	0.230

Tobacco leaves dipped in 0.01 M isotope solution ($N^{15}H_4Cl$) for 15 min, then rinsed and incubated in the dark for 4 hrs. All fractions prepared from the same homogenate.

SMALLER PARTICLES: In all the experiments so far described, the crude particles had been centrifuged out of the original homogenate at about 1,000 × g, and contained whole chloroplasts and mitochondria as well as a few fragments. To find out if the method could be used to prepare purified chloroplast fragments as well as whole ones, crude particles were taken from successive centrifugal fractions. The corresponding bottom and top pellets and middle fluid from spinach crude particles brought down at 500 × g, from 500 to 1,000 × g, and from 1,000 to 10,000 × g are shown in table V. The behavior of particles initially sedimenting at 500 × g and from 500 to 1,000 × g is the same as previously noted; top pellets are rich in chlorophyll and low in catalase activity, and the reverse is true for bottom pellets. The fractionation by density appears to fail for the smallest particles,

TABLE V

FLOTATION OF SMALL, NON-CHLOROPLAST PARTICLES COMPARED TO SEDIMENTATION OF LARGER NON-CHLOROPLAST PARTICLES

FRACTION	ORIGINAL PARTICLE SEDIMENTATION *					
	CATALASE/MG N **			CHLOROPHYLL/MG N		
	500 × g	1,000 × g	10,000 × g	500 × g	1,000 × g	10,000 × g
Crude particles	0.53	4.13	3.82	0.80	0.72	0.39
Bottom pellet	1.57	6.32	2.45	0.52	0.31	0.23
Fluid	0.63	0.68	1.70	0.71	0.81	0.59
Top pellet	0.04	0.37	1.10	1.14	1.08	0.56

* All crude particle fractions collected from the same tobacco leaf homogenate by successively faster centrifugations as indicated. Top pellet separated from the smallest original particles is not homogeneous chloroplastic material, as indicated by the low chlorophyll/N ratio and high catalase activity.

** Catalase as millimoles of perborate destroyed in 5 min.

TABLE VI

FAILURE OF YOUNG TOBACCO CHLOROPLASTS TO FLOAT IN A FLUID DENSITY 1.17 GM/ML

DENSITY OF SUSPENDING FLUID	CHLOROPHYLL IN BOTTOM PELLET AS % OF TOTAL		
	MATURE, GREEN LEAVES	YOUNG, GREEN LEAVES	YOUNG, ETIOLATED LEAVES
1.17	19	86	76
1.19	8	75	57
1.21	8	42	33

Young leaves were picked when less than ⅓ mature size. The crude particles for this experiment were sedimented out of their respective homogenates at 1,000 × g for 12 min.

which originally sedimented between 1,000 and 10,000 × g. The catalase activity is only twice as high in the bottom pellet as in the top, as opposed to the twenty-fold or more difference for the particles sedimenting at 500 or 1,000 × g. Further, the chlorophyll/N ratio of the top pellet in the case of the smallest particles is only 0.56, indicating much more impure chloroplast material than in the top pellets from the first two fractions where the ratios are 1.08 and 1.14. Analysis of a similar small particle top pellet for nucleic acid showed 25 μgm P/mg N, compared with 30 μgm P/mg N in the bottom pellet (table III, experiment 9). This again shows that this top pellet does not consist of pure chloroplast material. It therefore seems that the smaller particles include non-chloroplast material which also floats under these conditions.

YOUNG CHLOROPLASTS: Tobacco leaves one third grown or less were used as a source of young chloroplasts (12). Some plants were kept in the dark and the partially etiolated young leaves were homogenized for a source of young chloroplasts. In all cases, it was found that the chloroplasts from young leaves did not float as readily as did plastids from mature leaves. In table VI, the results of one experiment show that it takes a density of 1.2 or more to cause 50 % of the young plastids to float; whereas 80 % of the mature plastids float at density 1.17.

DISCUSSION

The separation according to particle density achieved by this method is seen to yield at least one reproducible and uniform fraction—that of the top pellet. This fraction always has a high chlorophyll/N ratio, a vanishingly low catalase content, and a uniformly low and equivocal nucleic acid content. These criteria, plus the small N^{15} incorporation into this fraction (Jagendorf, Erickson, and Wildman, unpublished) all characterize it quite firmly as consisting of chloroplasts in a very pure state.

A more elegant and all-inclusive fractionation of particles according to density could undoubtedly be achieved by using a density-gradient column, and separating particles from several regions of differing density (3, 4, 8). A tube with density gradient was used in preliminary experiments of this study, but found to be inadequate after centrifuging in the Spinco angle-head rotor. The proper application of the density-gradient method seems to require a horizontal high speed centrifugation.

The separation of pure chloroplasts by this method is possible only after a preliminary sedimentation at low speed. If all the particles in a homogenate were put into the dense medium, the chloroplasts would be contaminated with very small, catalase and nucleic acid containing, non-plastid particles. These are discarded with the supernatant, however, after a centrifugation at 1,000 × g. The density method does serve to remove presumably larger, denser mitochondria from chloroplasts. That there may exist numerous types of mitochondria, with differing densities, is seen from the work of Kuff and Schneider (8).

The procedure described here may prove useful in isolating large quantities of very pure chloroplast material. The separation is complete in one operation and involves fewer losses than does fractional centrifugation. The density technique also readily removes the unknown, white crystalline material found in preparations of tobacco chloroplasts, which could never be entirely eliminated by low speed fractional centrifugations (7). Finally, the combination of repeated centrifugal washing followed by density separation can be used to insure the highest possible degree of chloroplast purity, since the two methods separate chloroplasts by using different properties of the plastids.

Using this method, previous conclusions as to the composition of isolated chloroplasts have been fully confirmed. With either method of purification, when an impure but green preparation is separated to yield a fraction with the highest possible chlorophyll/N ratio, (i.e., pure chloroplasts), this fraction has almost no catalase activity and very little or possibly no nucleic acid. The complementary fraction (bottom pellet), with a much lower chlorophyll/N ratio is seen to be much richer in catalase and nucleic acid.

The cause of very low density of the chloroplasts is probably their high lipid content (11). Unfortunately, the proportion of lipid seems to be variable, depending on the plant material used. In spinach preparations, 90 % or more of the chloroplasts floated every time in a medium of density 1.17. With tobacco leaf preparations, from 60 to 90 % of the chloroplasts usually floated. Using red kidney bean leaves, a much greater degree of variability has been found, with from 5 % to 90 % of the chloroplasts floating on different occasions. We have observed that the chloroplasts of bean tend to float if the plants have been grown at high temperatures, and to sink if the plants were grown under cooler conditions. Facilities are not available to test this phenomenon more critically at the present time.

The failure of young chloroplasts to float until higher densities are used means that this method is not adequate to prepare pure, growing chloroplasts.

At the densities needed to float a majority of the young chloroplasts, many non-plastid particles would also float, as indicated by rising catalase activity and lower chlorophyll/N ratios in the top pellets.

Summary

A method is described for suspending leaf subcellular particulates in a dense medium and separating the particles that float from the ones that are sedimented after high speed centrifugation. Evidence is presented that chloroplasts and chloroplast fragments float to the top in a highly purified state. Some possible uses of this method for chloroplast purification are discussed.

It is found that young, growing chloroplasts do not float under these conditions, and may be more dense than mature plastids.

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ENZYMATIC AUXIN INACTIVATION BY EXTRACTS OF THE FERN OSMUNDA CINNAMOMEA L.[1,2]

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Because of the importance of auxins in the growth and metabolism of plants, many investigations have been conducted on the nature of enzymatic systems which inactivate both native auxins and synthetic indole acetic acid (IAA). The present paper describes the enzymatic auxin-inactivation system found at cut surfaces and in tissue extracts of the cinnamon fern *Osmunda cinnamomea* L. This system was first noted by Steeves et al (10) when they found that it interfered with accurate measurement of auxin diffusing from developing fronds of Osmunda. The relative activity of this enzyme system in extracts of leaves harvested at different stages of growth, from dormancy to maturity, and in extracts of different parts of developing leaves will be described. The possible role of two well-known enzymes in the auxin destruction process will be discussed. A consideration of the possible physiological significance of the enzyme system described below, in the light of its distribution in growing fronds, and its activity in fronds of different ages will be reserved for a second paper.

Since Thimann (14) noted that leaf extracts of *Vicia faba* and *Helianthus annuus* would inactivate native auxins in solution, a considerable body of literature on auxin destruction has accumulated. For a full review of the subject, see Tang and Bonner (12) and Gordon (4). The nature of the auxin inactivation system has been a matter of controversy for a number of years. Thimann (14) and Wetmore and

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Morel (19) felt that a polyphenol oxidase system was involved. Van Overbeek (15) suggested that the system found at least at cut surfaces in some strains of corn was due to some sort of catalase-peroxidase balance in the tissues. Enzymatic IAA-destruction systems appear to be found in extracts of many higher plants. IAA-oxidases have been noted or described from peas (12), carrots, lettuce, cabbage, spinach, Avena (13), yellow wax bean roots (17), Phaseolus seedlings (7) and pineapple (5). The system found in pea extracts was considered, on the basis of inhibitor studies, to include an iron enzyme (12), which was thought to be a peroxidase (3). Wagenknecht and Burris (17), from similar studies, concluded that the system in bean roots involved a copper enzyme, while Gortner and Kent (5) found that the auxin destruction by extracts of pineapple tissue was not inhibited by strong copper chelating agents such as 8-hydroxyquinoline. Thus, at present, it is impossible to generalize about the existence of a single auxin destruction system which is found in all plant material investigated.

Materials and Methods

Living material of the fern *Osmunda cinnamomea* was collected from nature and used directly, or stored in a deep freeze until needed; or collected from plants which were stored in a cold room at 4° C until grown in the greenhouse. In each experiment, plant material from only one of the above treatments was used, and comparable plants were selected. In experiments involving enzyme extracts, tissue was ground with purified sand in distilled water, buffer, or a suitable solution of inhibitor or other reagent to be tested, then either filtered through Pyrex wool or centrifuged at $2500 \times g$ for 10 minutes. The filtrate or the supernatant was used as the enzyme preparation. The final reaction mixtures were made up to the equivalent of 1 gm fresh weight of tissue per 3 ml of solution, except where otherwise noted. Reaction mixtures were incubated for a suitable period of time at room temperature (roughly 23° C, excepting table IV), and then mixed with an equal volume of 3 % Bacto-agar at 100° C. The agar was allowed to harden, and was then cut into blocks $2.67 \times 2.75 \times 1.5$ mm. The blocks were assayed for auxin by means of the standard Avena test (18).

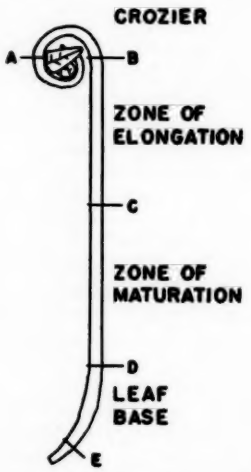

Fig. 1. Diagram of young developing frond of *Osmunda cinnamomea*. No pinnae are yet set off below crozier. (A, B, C, D, E) Separate fronds were cut at these points for extraction and diffusion experiments.

Peroxidase was prepared from horseradish according to the technique of Elliott (1). The preparation used had a Purpurogallin Zahl (PZ) of 300. Peroxidase activity of tissue extracts was determined by the technique of Willstätter and Stoll (20), modified by Sumner and Gjessing (11), and expressed as PZ per gm fresh weight of tissue. Catalase activity of extracts was determined immediately on fresh extracts according to the technique of von Euler and Josephson (16), and the results given by the expression Katalase Fähigkeit (Kat.F.) $\times 10^3$ per gm fresh weight of tissue.

Experimental Results

Auxin Inactivation by Extracts of Leaves and Rhizomes: In the normal pattern of development for the fern *Osmunda cinnamomea*, leaves require almost four years, involving 5 growing seasons, to develop, from initiation to final maturity (9). These studies are confined to the final stage of development of leaves during the fourth year. At the beginning of the 5th season, those leaves which will become mature during that season are dormant, within a winter bud. Their tips are coiled through at least three complete revolutions, forming a crozier, and all of the pinnae are present at least as primordia (9). During the fifth growing season, after an obligate period of dormancy, each leaf rachis begins to elongate rapidly, the crozier uncoils, and the pinnae expand. At this time (see fig 1), each partially developed frond has a part of the rachis within the crozier which has not yet begun its final elongation (except for the very slight differential elongation which brings about uncoiling), a part just below the crozier which is elongating rapidly, a part in which elongation is complete and the tissues are maturing, and a part, the leaf base, which undergoes no elongation at all during this time (8). It is possible to separate these four regions fairly accurately by marking the rachis with India ink, and recording the progress of the wave of elongation.

Initial experiments were performed to determine the relative activity of enzymatic IAA-inactivation by water extracts of whole leaves in different stages of development. Leaves were cut off at ground level, i.e., just above the leaf base, and extracted in distilled water as described above. Data from two experiments are shown in table I, showing the IAA removed from reaction mixtures during a 12- or 16-hour incubation with leaf extracts at 23° C. These results, and those shown below, express the activity of 1 gm

fresh weight of tissue per 3 ml of reaction mixture. Extracts of fronds just emerging from the bud show the greatest amount of activity, completely destroying IAA at concentrations of 500 μgm/l in that time. Auxin-destruction activity remained fairly high in extracts of these plants until the fronds were between 16 and 20 cm high, and then dropped off sharply and rather irregularly until extracts of fronds 60 cm high showed no activity at all. Though not included in table I, data from several other experiments show that extracts of fronds which are still dormant, and within the bud, inactivate IAA at a considerably slower rate than those of fronds which have broken dormancy. In one case, an extract of dormant leaves incubated for 20 hours with 400 μgm/l IAA reduced Avena curvatures from 24.5° ± 2.7 (maximum for the day) to 10.4° ± 1.1. Extracts of medium-sized, growing leaves reduced curvature to 0.6° ± 1.2, destroying virtually all of the added IAA, while extracts of mature leaves did not reduce curvature at all, giving 26.4° ± 2.3 in bioassay. These experiments indicate that activity of the auxin-destroying system is low in extracts of dormant leaves, rises to a high peak during the early stages of growth, and falls off almost entirely as the leaves pass through their maximum growth period and become mature. Also not included in table I, but confirmed on four separate occasions, are data showing a complete lack of destruction of added IAA by water extracts of rhizome tissue. In one case, incubation of 125 μgm/l IAA for 12 hours produced 29.0° ± 1.9 of curvature, while the control value was 29.9° ± 0.9. (Maximum curvature for the day was 34.0° ± 0.7.)

Data from further experiments in vitro on the separated portions of partially developed fronds (table II) show that the activity of the enzyme system is highest in extracts of the youngest tissue of the leaf, the crozier, tapering off in the zone of elongation, and dropping further in the lower tissues, the zone of maturation and the leaf base. Reactions were run for 0, 2.5 and 5 hours, and were stopped by immersing the tubes containing the reaction mixtures in boiling water for 5 minutes.

TABLE I

VARIATIONS IN THE ABILITY OF WATER EXTRACTS OF WHOLE FRONDS OF OSMUNDA CINNAMOMEA IN DIFFERENT STAGES OF DEVELOPMENT TO DESTROY ADDED IAA. TWO SEPARATE EXPERIMENTS. AMOUNT OF IAA DESTROYED CALCULATED FROM AVENA CURVATURE. EXTRACTS ALONE GAVE NO CURVATURE

DESCRIPTION OF FRONDS	INITIAL CONC IAA, μGM/L	IAA DESTROYED	
		μGM/L	% INITIAL CONC
Just emerging from bud, 4–6 cm tall	500	500 *	100
14–16 cm tall	500	484 *	97
About to set off pinnae, 20 cm tall	500	407 *	82
8 pairs pinnae set off, 40–56 cm tall	500	< 250 *	< 50
14–16 cm tall	500	500 **	100
Half uncoiled, 60 cm tall	250	0 **	0

* 16 hr incubation at 23° C.
** 12 hr incubation at 23° C.

TABLE II

IAA REMAINING IN REACTION MIXTURES AFTER INCUBATION WITH WATER EXTRACTS OF PARTS OF ACTIVELY DEVELOPING FRONDS OF OSMUNDA CINNAMOMEA

PART EXTRACTED	HRS INCUBATED		
	0	2.5	5
	μgm/l	μgm/l	μgm/l
Crozier and pinnae	125	56	37
Zone of elongation	125	108	97
Zone of maturation	125	140	136
Leaf base	125	113	114

Initial conc of IAA 125 μgm/l. Residual auxin calculated from Avena curvatures. Extracts alone gave no curvature. One gm tissue per 3 ml reaction mixture.

THE NATURE OF THE ENZYME SYSTEM: Preliminary experiments indicated that inactivation of added IAA could be completely prevented by boiling the extract for 5 minutes. This heat lability of the system suggested that it was enzymatic in nature. Further work showed that auxin destruction by the system could be at least partially prevented by carrying out the reaction in an atmosphere of nitrogen, as shown in table III, suggesting that the system was oxidative.

The effects of various enzyme inhibitors were investigated. In these experiments, fronds 12 to 35 cm tall were ground with sand in a solution of the inhibitor to be tested, filtered through Pyrex wool, made up to volume with a solution of the appropriate inhibitor, and IAA added to the final concentrations shown in table III. Reaction mixtures were incubated for 1, 5 or 6 hours, and in each case, a control, containing just the inhibitor and IAA, was included to determine the effect of the inhibitor on the Avena plants. Since the several auxin concentrations used give maximum curvature, it is impossible to express results as percentage inhibition, and they are given simply as degrees of Avena curvature. The auxin-destruction system is known to be cyanide sensitive (10) and the results of several experiments with other inhibitors are shown in table III. All inhibitors were used at a final concentration of 10^{-3} M. Inhibition of the system by cyanide and by sodium azide and lack of inhibition by hydroxylamine hydrochloride indicate the presence of a heavy metal in the system. Furthermore, only partial inhibition by phenylthiourea and variable results with thiouracil, plus a complete failure of inhibition by sodium diethyldithiocarbamate suggest that iron rather than copper is the heavy metal involved (6).

PEROXIDATIVE AUXIN DESTRUCTION BY OSMUNDA EXTRACTS: Since it has been suggested that the enzyme system in peas involves, at least in part, a peroxidative component (2, 3), experiments were

TABLE III

EFFECTS OF VARIOUS TREATMENTS AND INHIBITORS ON AUXIN DESTRUCTION BY WATER EXTRACTS OF FRONDS OF OSMUNDA CINNAMOMEA 6 TO 16 CM TALL *

TREATMENT OR INHIBITOR	INITIAL CONC IAA, μGM/L **	HRS INCUBATION, ROOM TEMP	DEGREES AVENA CURVATURE IAA + INHIBITOR	DEGREES AVENA CURVATURE IAA + INHIBITOR + ENZYME, AFTER INCUBATION
None	800	6	20.0 ± 2.5	0.7 ± 0.46
N atmosphere	800	6	20.0 ± 2.5	19.1 ± 1.2
None	250	5	19.6 ± 1.1	3.2 ± 1.4
None	100	1	21.1 ± 1.3	0
$NH_2OH \cdot HCL$ .001 M	250	5	27.4 ± 1.8	2.8 ± 0.76
$NH_2OH \cdot HCL$ .001 M	100	1	20.6 ± 3.2	5.9 ± 0.83
Thiouracil .001 M	250	5	21.8 ± 0.75	24.0 ± 1.4
Thiouracil .001 M	100	1	25.3 ± 2.1	5.3 ± 0.81
Sodium azide .001 M	250	5	21.7 ± 1.5	23.6 ± 1.3
Sodium diethyldithiocarbamate .001 M	250	5	22.6 ± 0.78	3.4 ± 1.0
Phenylthiourea .001 M	250	5	24.5 ± 1.4	11.3 ± 0.5
None	250	5	22.2 ± 2.2	6.2 ± 0.62
Boiled extract †	250	5	17.0 ± 0.72	13.3 ± 0.56

* Fronds used in nitrogen experiment were over 30 cm tall, with 8 pairs of pinnae set off.

** Concentrations in blocks assayed are 0.5 concentrations in reaction mixtures because of a 1 : 1 dilution with 3 % agar.

† From fronds just breaking dormancy 0.25 gm/ml reaction mixture.

Enzyme preparation 0.33 gm/ml reaction mixture. Each IAA conc represents a separate experiment.

undertaken to determine the effect of the addition of H_2O_2 on IAA inactivation by Osmunda leaf extracts. Reaction mixtures initially contained leaf extract representing 0.25 gm fresh weight of dormant leaves per ml, 123 μgm/l IAA (7×10^{-7} M), and 7×10^{-6} M H_2O_2. Aliquots were removed at 0, 3 and 6 hours, and were immediately put into tubes and allowed to remain in boiling water for 5 minutes. They were then made into agar blocks, and stored in the cold (4° C) until assayed. The reactions were run in the dark, at 30° C. Dialysed and non-dialysed extracts were tested, both with and without H_2O_2. Suitable controls, containing IAA alone and IAA plus H_2O_2, were included to determine whether or not differences observed could be accounted for by non-enzymatic destruction of IAA by the peroxide. The results of one such experiment are shown in table IV. It may be seen that the addition of H_2O_2 somewhat stimulated the rate of IAA destruction by the crude extract. Furthermore, the dialysed extract destroyed no IAA in the absence of peroxide, suggesting that during dialysis, either a substrate for some system producing H_2O_2 or even H_2O_2 itself was removed, or else the peroxide originally present in the extract was broken down by catalase during dialysis.

TABLE IV

INACTIVATION OF IAA BY CRUDE AND DIALYSED EXTRACTS OF DORMANT OSMUNDA FRONDS, WITH AND WITHOUT ADDED H_2O_2

EXTRACT OR CONTROL	INCUBATION PERIOD, HRS	IAA IN DEGREES AVENA CURVATURE	INACTIVATION IN DEGREES CURVATURE
Crude	0	23.6	
	3	17.7	
	6	18.9	4.7
Crude + H_2O_2	0	26.2	
	3	18.0	
	6	16.2	10.0
Dialysed	0	23.9	
	3	22.8	
	6	23.8	0.1
Dialysed + H_2O_2	0	25.3	
	3	22.7	
	6	12.0	13.3
IAA		26.3	
IAA + H_2O_2	0	23.2	
	6	25.1	

Initial conc of IAA was 7×10^{-7} M (123 μgm/l). Initial conc H_2O_2 was 7×10^{-6} M.

VARIATIONS IN PEROXIDASE AND CATALASE ACTIVITY IN LEAF EXTRACTS DURING LEAF DEVELOPMENT†: Since a large variation was noted in the activity of the auxin destruction system in leaf development, and it appeared that a peroxidase was at least in part involved, the activity of peroxidase and catalase during leaf development was investigated. The enzyme assays were made from whole fronds, cut at ground level, just above the leaf base, and ground immediately in a 0.1 *N* solution of Na_2HPO_4. Peroxidase activity is expressed as PZ per gm fresh weight of tissue. Catalase activity was determined at once and the results are given by the expression Kat.F. $\times 10^3$ per gm fresh weight of tissue. The results of these assays are shown in table V. Catalase activity is relatively low in the very young frond (Kat.F. $\times 10^3$ = 3), and increases rather slowly at first, as the leaf

TABLE V

VARIATIONS IN CATALASE AND PEROXIDASE ACTIVITY OF BUFFER EXTRACTS OF LEAVES OF OSMUNDA CINNAMOMEA IN VARIOUS STAGES OF DEVELOPMENT

DESCRIPTION OF FRONDS	LENGTH, CM	FRESH WT, GM	AGE, DAYS FROM DORMANCY	KAT.F. $\times 10^3$ PER GM FRESH WT	PZ PER GM FRESH WT
Dormant	4.0	17.0	0	...	< 0.1
Just breaking dormancy	6.5	0.8	3	3	0.15
Uncoiling, about to set off pinnae	45.0	10.2	9	10	0.30
14 pairs pinnae set off	67.2	46.0	14	40	0.40
Uncoiling just completed	92.0	40.0	21	60.5	0.35
Adult leaf fully expanded	90.0	60.0	47	610	0.40

develops, since at the end of 21 days, when the frond is entirely uncoiled, the value is only 60.5. Well after the frond has matured and growth has ceased, 47 days from the time the frond broke dormancy, the value has increased to 610, almost 200 times the initial value. Although peroxidase activity is relatively low at the start of growth ($PZ < 0.1$) it reaches a relatively high value ($PZ = 0.4$) fairly early during development and maturation. However, this value represents only a three- to four-fold increase over its initial value.

If this peroxidase activity is an important factor in auxin destruction by the tissue extracts, the results shown above offer a partial explanation for the wide variation in auxin inactivation by extracts of fronds of different ages. In dormant or very young fronds, catalase activity is low, but peroxidase activity is also low, and the rate of auxin destruction might be expected to be low also. Almost immediately, however, peroxidase activity rises, as does the rate of auxin destruction. Catalase activity rises steadily, however, and perhaps competition with peroxidase for H_2O_2 becomes limiting. If this were so, the rate of auxin destruction would drop as catalase activity increased. Other substrates for peroxidase, present in the extract, and an inhibitor, mentioned below, however, undoubtedly complicate the picture.

EXISTENCE OF AN INHIBITOR OF AUXIN DESTRUCTION IN LEAF EXTRACTS: Reports from the literature (5, 12) indicate that many tissues are rich sources of a naturally occurring inhibitor of auxin destruction. There appears to be such an inhibitor in water extracts of Osmunda leaves. If a boiled extract of fronds just breaking dormancy is added to an active preparation of IAA-oxidase from young leaves, with added IAA, the rate of destruction of IAA during a 5-hour period is significantly lower than that of a control, without the boiled extract (table III). Since it has been shown (3) that horseradish peroxidase will inactivate IAA in the presence of H_2O_2, the influence of a similar boiled extract on IAA inactivation by horseradish peroxidase was investigated. IAA destruction by 0.1 mg per ml horseradish peroxidase, with added peroxide, was completely inhibited over a two-hour period by the addition of 0.25 gm per ml reaction mixture of boiled extract, while a control, without the boiled extract, completely destroyed 105 μgm/l IAA, the original concentration, in the same time. Hence, there is a heat-stable factor which inhibits not only the native enzyme preparation, but also horseradish peroxidase, from inactivation of IAA. The activity of this factor throughout leaf development is unknown at present. However, it suggests one more cause for the variability of extracts of comparable fronds and fronds of different ages to destroy auxin. The boiled extract with auxin alone, after incubation, did not reduce Avena curvatures below those of the auxin controls by a significant amount.

SUMMARY

An enzyme system which inactivates auxin at cut surfaces and in crushed tissue extracts of fronds of the fern *Osmunda cinnamomea* L. is described. Its activity in extracts of different tissues, and extracts of leaves of different ages is noted. Rhizome extracts fail to destroy IAA. The system is heat labile, involves a heavy metal, probably iron, and requires oxygen. Results obtained from the addition of H_2O_2 to the Osmunda system suggest a peroxidase to be involved. The enzyme system appears to be similar in many respects to that obtained from peas.

Variations in catalase and peroxidase activity in extracts from developing fronds are described, and evidence suggesting an inhibitor of auxin inactivation is presented. These factors may be partially responsible for variations observed in auxin destruction by extracts of fronds of different ages.

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A COMPARISON OF AUXIN DESTRUCTION BY TISSUE EXTRACTS AND INTACT TISSUES OF THE FERN OSMUNDA CINNAMOMEA L.[1,2]

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Although much work has been done on the nature of enzyme systems which inactivate native auxins and synthetic indole acetic acid (IAA) (5, 6, 10, 11, 15), most of the experiments have been carried out with plant extracts, and many of them were done using non-physiological concentrations of IAA. There are few in vivo studies on the physiological function of such systems. Tang and Bonner (11) have suggested that in normally grown peas, the system found does not destroy auxin within the plant, while it does appear to be functional in etiolated seedlings. Von Abrams (14) found that growth differences between tall and dwarf etiolated pea seedlings could not be correlated with IAA-oxidase activity in tissue extracts of the two varieties. Other experiments involving tissue extracts (4) or auxin diffusion across a cut surface into agar blocks (2, 13) do not convincingly separate the phenomenon of enzymatic auxin destruction by damaged tissue from destruction by living and intact cells. The last three papers (2, 4, 13) show an inverse correlation between growth of tissues and activity of the destruction system, either in tissue extracts or at cut surfaces. Only Van Overbeek (13) attempted to demonstrate a direct causal relationship between the two phenomena. An earlier paper from this laboratory (9) has reported auxin destruction at cut surfaces in *Osmunda cinnamomea* L. In presenting a method for overcoming this inactivation during auxin diffusion studies, the authors considered it to be a cut surface phenomenon only. A second paper (1) has studied the distribution of this system as found in crushed tissue extracts of various parts of the plant, and has dealt with some of its characteristics. The present paper will consider further the physiological significance of this auxin destruction system, and will present evidence which suggests that auxin is not destroyed in the non-growing tissue of this plant, but disappears only in parts which are growing. The somewhat anomalous behavior of the rhizome tissue will be discussed.

MATERIALS AND METHODS

Plants of the fern *Osmunda cinnamomea* were either collected in the spring and used directly, or collected in the fall, kept in a cold room at 4° C for an appropriate period of dormancy, and then grown in a greenhouse the following spring. All of the experiments described below were done on fronds in their fourth and final year of development (1). In

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production and transport experiments, auxin was permitted to diffuse from tissues into 1.5 % agar blocks (8 × 11 × 1.5 mm). These experiments were done at approximately 23° C in a closed chamber lined with wet paper towels to keep humidity high and minimize wilting. Except where otherwise noted, the cut surfaces of tissues were treated with 0.005 M KCN to prevent cut surface auxin inactivation, following the technique described by Steeves et al (9). In experiments involving transport of externally applied IAA, an emulsion of 0.5 % IAA in 50 % each of lanolin and water was used. Ether extractions were made from tissue slices roughly 1 mm thick, at 0° C, for 4 hours, with freshly distilled, peroxide-free diethyl ether. The extracts were then evaporated to dryness over a steam bath, and made up to appropriate volume with agar to a final concentration of 1.5 %. For all auxin bioassays, agar was finally cut into blocks 2.67 × 2.75 × 1.5 mm and assayed by means of the standard Avena test (16). Crude preparations of the IAA-inactivating enzyme system were made as described in a previous paper (1).

NORMAL DISTRIBUTION OF AUXIN IN THE GROWING FROND: In a series of experiments to be published elsewhere, it was shown that all of the auxin measurable by the technique used was produced by the pinnae of developing fronds of Osmunda, and not by the rachis (8). Thus, by removing all of the pinnae from a developing frond, or removing the portion of the rachis bearing the pinnae, it was possible to obtain parts of the rachis in which there was no detectable auxin present at the start of a given experiment. It was also shown that transport either of naturally occurring auxin or of IAA applied in a lanolin-water emulsion to the epidermis was strictly polar, through sections of elongating or mature rachis tissue.

Experiments were then undertaken to determine the course of disappearance of endogenous auxin from the frond rachises during development. Developing fronds were selected on which no pinnae were as yet set off below the crozier, and on which there were

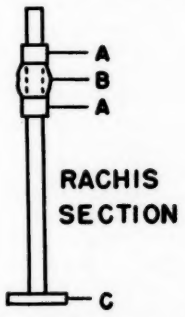

FIG. 1. Method for applying IAA-lanolin-water emulsion to a given area of epidermis of rachis section of Osmunda frond. A. 5 mm sections of rubber tubing. B. Emulsion filling gap between tubes. C. Agar block at basal end of rachis section. Agar block was 2, 4 or 6 cm from center of lanolin.

TABLE I

DISAPPEARANCE OF ENDOGENOUS AUXIN PRODUCED BY PINNAE FROM RACHIS TO RHIZOME

POINT OF RACHIS AT WHICH AUXIN WAS COLLECTED *	EXPERIMENT 1	2	3	4	5
180° apical from base of crozier	...	...	...	...	15.3
Base of crozier	19.3	21.9	19.9	15.6	16.5
Base of zone of elongation	8.9	8.7	10.0	3.9	...
Base of zone of maturation (ground level)	11.5	6.7	13.4	3.1	7.7
⅔ distance from ground level to attachment to rhizome	10.3	9.0	...	...	...

Auxin collected by diffusion. Data expressed as degrees of Avena curvature. Cyanide technique used throughout. Each diffusion from two fronds tied together. Pairs of fronds in each experiment roughly comparable.

* See (1), fig. 1: A, B, C, D and E.

no pinnae on the outer 180° of the crozier (see (1), fig 1). The fronds were cut off just above the first 180° of crozier; or just below the crozier, but above the zone of elongation; or just at the base of the zone of elongation; or at ground level, just above the white leaf base; or at a level 2/3 of the distance between ground level and the attachment of the leaf to the rhizome, a point normally below the ground. The positions of these cuts are shown by the letters A, B, C, D and E, in (1), figure 1. The level dividing the zone of elongation from the zone of maturation was determined fairly accurately by marking the developing rachis at regular intervals with India ink, and recording the progress of rachis elongation through the relative separation of the marks. It was found that the rachis tissue within the crozier elongated only slightly, though enough to bring about uncoiling, while the major elongation was confined to a region just below the uncoiling pinnae-bearing crozier. Auxin was collected from the tissue distal to the cut in each case, and separate fronds, tied in pairs, were used for each individual diffusion. The results of five such experiments are shown in table I. Comparable results were obtained in numerous other experiments. The only region in which a significant disappearance of auxin takes place is the zone of elongation. The amount of auxin travelling through the rachis below the auxin source, the pinnae, but still within the crozier and above the zone of elongation, remained constant within the limits of variability of the material. There is a significant drop in the amount of diffusible auxin from the top to the bottom of the zone of elongation, but there appears to be no drop at all through the entire remainder of the rachis and leaf base. Thus, the amount of endogenous auxin obtainable by diffusion, using the cyanide technique, does not drop in any tissue of the rachis that is not actually growing.

THE DISTANCE WHICH CYANIDE TRAVELS ABOVE

THE POINT OF APPLICATION IN CONCENTRATIONS EFFECTIVE IN PREVENTING CUT SURFACE AUXIN INACTIVATION: In view of the fact that cyanide is such a powerful physiological agent, even at the low concentrations used in these experiments, it was considered desirable to determine just how far above the original cut surface, to which it was applied, cyanide was still effective in preventing cut surface auxin inactivation. Eight fronds, almost fully uncoiled, but still producing considerable auxin, were cut off roughly 10 cm below the zone of elongation, and diffusion experiments were done in the following manner: the fronds were matched into comparable pairs, tied together, a fresh cut made at the basal end, and 0.01 ml of 0.005 M KCN applied to the cut surfaces of each pair. Agar blocks were then placed on the cuts and left for 2 hours. At the end of this time the blocks were removed. On the first pair of fronds, 0.01 ml of water was applied to the original cut surface, and a second block applied for 2 hours. On the second pair, the procedure was repeated, after a new cut had been made 1.5 mm above the original, before applying the water. On the third and fourth pairs, the procedure was repeated, but the fresh cut was made 5 and 10 mm respectively above the original. All eight blocks were then assayed for auxin, and the results are shown in table II. At 1.5 mm above the original cut surface, to which KCN was applied initially, inhibition of cut surface auxin inactivation was still complete, and the second block contained as much auxin as the first; 5 mm above the original cut, the cyanide was less effective, as shown by the lower yield of auxin obtained from the second block; 10 mm above the original cut, there was not enough cyanide to affect the destruction system at all, so that almost no auxin was obtained from the second block. In the second experiment shown in table II, the second cuts were made 1 and 2 cm respectively above the original, previous to the second diffusion, and it was evident that cyanide was ineffective at those distances since the blocks from the second diffusions contained no auxin at all. Thus,

TABLE II

EFFECTIVENESS OF KCN IN PREVENTING CUT SURFACE INACTIVATION OF ENDOGENOUS AUXIN AT DIFFERENT DISTANCES ABOVE ORIGINAL CUT SURFACE TO WHICH 0.01 ML OF 0.005 M KCN WAS APPLIED. DATA FROM TWO EXPERIMENTS. 0.01 ML H_2O ADDED TO CUT SURFACE FOR SECOND DIFFUSION

FROND PAIR	DISTANCE SECOND CUT ABOVE FIRST	IAA COLLECTED 1ST 2-HR DIFFUSION	IAA COLLECTED 2ND 2-HR DIFFUSION
	mm	*Degrees Avena curvature*	*Degrees Avena curvature*
1	0	11.9	13.2
2	1.5	13.0	13.4
3	5	22.6	16.3
4	10	10.4	2.4
1	0	11.8	11.2
2	10	11.7	0
3	20	12.2	0

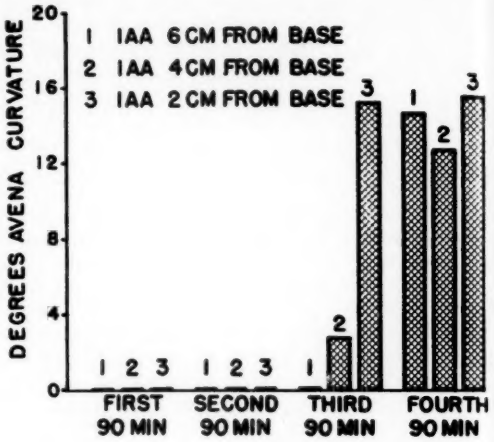

FIG. 2. IAA collected during four consecutive 90-min periods from the bases of sections of the zones of maturation of 3 leaves of *Osmunda cinnamomea*, to which IAA was applied in a lanolin-water emulsion 2, 4 and 6 cm respectively above the bases. Details in text.

under these conditions, cyanide does not move up into the rachis more than 1 cm in 2 hours, at least in concentrations strong enough to inhibit cut surface auxin inactivation.

TRANSPORT OF EXTERNALLY APPLIED AUXIN BY SECTIONS OF MATURE RACHIS: The polarity of auxin transport by the rachis made it possible to determine whether the inactivation is in intact tissue or only at cut surfaces, in the following way: the croziers and laminar tissue were removed from three comparable young fronds, removing the source of endogenous auxin. After 23 hours, or enough time to allow the endogenous auxin to disappear from the remaining rachises, the latter were cut off at ground level, two sections of rubber tubing 5 mm long were slipped over the rachis and placed 1 cm apart, with the center of the 1 cm gap respectively 2, 4 and 6 cm above the basal cut. The gap was then filled with a 0.5 % auxin lanolin-water emulsion, described above. After the application of KCN to the basal end, agar blocks were placed on the cut surface for auxin diffusion, and were replaced every 90 minutes over a period of 6 hours. A diagram of the experiment is shown in figure 1. The results of the assays of the agar blocks for auxin are shown in figure 2.

It was reasoned that since no auxin was produced in the rachis, and none appeared from the basal end during the first three hours, all auxin collected must have been IAA which had diffused into the transport system from the lanolin-water emulsion. Therefore, in the longest section, to which IAA had been applied 6 cm from the basal end, it should have taken it longer to reach the basal end than that applied 4 or 2 cm from the cut, and this is seen to be the case. Furthermore, since the surface area covered by the emulsion was constant in each case, the rate of auxin

diffusing into the tissue at the point of application should also be constant. Therefore, as soon as IAA was diffusing from the basal cut at a constant rate, the amount obtainable in any given length of time, here 90 minutes, should provide a measure of any endogenous destruction. If auxin is being destroyed by the living tissue, the further away from the point of application the auxin is collected, the lower should be the final steady rate of auxin diffusion from the cut surface. If, on the other hand, the tissue is not destroying auxin endogenously, the final steady rate of diffusion should be the same, regardless of the distance from the point of application to the point of collection. As shown in figure 2, the amount of auxin obtained during the fourth 90 minute period is approximately the same, regardless of the length of the transport path, and there is no indication of any endogenous destruction. These results do not preclude the existence of a small region at the base of each section, under the influence of cyanide in these experiments, which normally inactivates auxin within the tissue, but experiments shown below indicate that the destruction system is actually most active in extracts of the upper tissues, the crozier and the zone of elongation. Data from ether extractions, also shown below, rule out such a possibility as well.

A Comparison of Auxin Disappearance in Living Tissue and Auxin Inactivation by Extracts of the Identical Tissue: Since the auxin-destroying ability of leaf extracts varied widely (1), a comparison of in vivo auxin disappearance in rachis tissues and in vitro auxin inactivation by extracts of the identical tissues was made. Six young fronds 29 to 36 cm long, and with no pinnae set off below the croziers (see (1), fig 1) were matched into comparable pairs, and diffusion experiments were done as follows: auxin was collected just below the croziers in one case, just below the zone of elongation in the second, and at ground level, below the zone of maturation in the third (see (1), fig 1: B, C, D). The portions of the fronds below the cuts were left on the plants until later to prevent their wilting. After the diffusions were completed, all 6 croziers were combined and extracted in 0.067 M phosphate buffer, pH 6.1, as were the 6 pieces representing the zones of elongation and the 6 of the zones of maturation. The extracts were centrifuged at 2500 × g for 10 minutes and the supernatants were decanted, made up to volume with buffer, and stored overnight in the cold room at 4° C. The following morning, the extracts were incubated at 23° C with IAA at a final concentration of 125 μgm/ml with tubes for each extract in which IAA was added 5 and 0 hours before the tubes were immersed in boiling water for 5 minutes. The reaction mixtures were then made up into agar blocks and assayed for auxin, together with the agar blocks from the diffusions of the previous day. The data are shown in table III. The results of the diffusions agree with those shown earlier (table I); only in those tissues which are actively growing is there a significant disappearance of diffusible auxin. However, all three extracts destroyed added IAA, although the zone of maturation showed less activity, in vitro, than either the zone of elongation or the crozier. All cut surfaces which had been treated with cyanide were removed by severing and discarding the terminal centimeter (see table II). The data in table III indicate that the same tissue that transports endogenous auxin in vivo with no loss measurable by the Avena test, in this case the zone of maturation, has the ability to destroy added IAA in vitro, upon extraction.

Table III

Comparison of in Vivo Disappearance of Endogenous Auxin in Parts of Developing Fronds of Osmunda cinnamomea with in Vitro Destruction of Added IAA by Water Extracts of the Identical Tissues. Results of Diffusions Expressed as Degrees Avena Curvature (upper table). Residual Auxin in Reaction Mixtures Calculated from Avena Curvatures (lower table)

Level of Diffusion	Auxin yield, 3-hr diffusion *
Below crozier, above zone of elongation	19.9
Just below zone of elongation	10.0
Below zone of maturation above leaf base	13.4

Region of leaf extracted	Auxin added 0 hr	Residual auxin after 5 hr
	μgm/l	μgm/l
Crozier and pinnae	125	77
Zone of elongation	125	94
Zone of maturation	125	100

* Maximum curvature for the day 31.0°.

Auxin Content of Different Parts of Leaves and Rhizome, as Measured by Ether Extraction: Since measurement of auxin diffusing into or out of a given piece of tissue does not necessarily give an indication of the amount of auxin contained within the tissue, and therefore is not an infallible method for demonstrating the presence or absence of auxin destruction in vivo, it was necessary to make ether extractions of various parts of the leaves and rhizome, to determine the content of extractable growth substance in each. Furthermore, all diffusion experiments were done with cyanide, and it was important to know that the auxin obtained in this manner was really the amount that could be accounted for by diffusion techniques under normal circumstances, that is, that the cyanide was not poisoning a very mild endogenous destruction system, well above the cut surface to which it was applied. Plants with fronds 58 to 61 cm high were selected. At this time 3 or 4 pairs of pinnae were set off below the croziers of the fronds extracted. The croziers with pinnae attached, 10 cm sections of the zones of elongation, taken below the lowest pair of pinnae, 10 cm sections from the upper and lower portions of the zones of maturation, the white leaf bases, 5.5 to 7.5 cm long, and 1.5-cm sections of the rhizome, one directly below the point

of attachment of the current year's leaves, and a second 1.5 cm below the first, were all separately extracted with ether, as described above. All extracts were made up to a final volume of 5 gm fresh weight of tissue per ml 1.5 % agar, and assayed by the Avena test. In order to be sure that the auxin extracted was indeed just diffusible auxin, extracts were also made, in another experiment, from the lower zone of maturation and a 3-cm section of the rhizome, taken from just below the attachment of the current year's leaves, from plants from which the croziers and all laminar tissue had been removed 48 hours previously. The assays of these extracts were compared with assays of similar extracts from a comparable plant which was left intact until extraction. In both of these experiments, plants were selected on which the leaves were in a stage of maximum growth and high auxin production. The data from these bioassays are shown in table IV.

Since it was known that no auxin could be obtained by diffusion from rachises from which all laminar tissue had been removed 24 hours previously, it was determined that the auxin extracted was indeed diffusible auxin, since it also disappeared not only from the rachis, but from the rhizome as well, during the 48 hours following decapitation. The content of auxin in normal fronds was lowest in the crozier, which included the pinnae in which the auxin is produced. This auxin is funnelled into the rachis, in which the content of auxin per gram of tissue became higher. Down the entire length of the rachis, through the leaf base, there was no loss of extractable auxin, and in the rhizome, in which auxin from several leaves —in these cases, six—was accumulating, the content of auxin per gram of tissue was even higher.

From a study of diffusion data, one might expect to find the level of auxin per gram of tissue to be considerably higher in the zone of elongation than in the zone of maturation, since the amount of auxin entering the former in a given length of time is greater than that entering the latter. However, as shown in table IV, this is not the case. This apparent inconsisteney may be partially explained as follows: the average density of the zone of elongation, determined by measuring both fresh weight and volume of given sections, is roughly two times that of any portion of the zone of maturation. Thus, in order to obtain equal weights of tissue from these two regions, one must have roughly twice the volume of tissue from the zone of maturation as from the zone of elongation. Therefore, if one were to extract equal volumes of these two tissues instead of equal weights, one would find approximately twice as much auxin per unit volume in the zone of elongation as in the zone of maturation. Since the density of the zone of maturation does not change down to the leaf base, the figures given in table IV for regions of rachis below the zone of elongation are valid on both a weight and volume basis. As the reasons for density changes in growing tissue are highly complex, and data on rates of auxin transport are not yet available for this material, the seeming inconsistency mentioned above will not be considered further in this paper.

The data obtained from these extractions show no decrease in the amount of extractable auxin below the zone of elongation, and furthermore, an accumulation of auxin in the rhizome. This evidence, then, supports the conclusions obtained from diffusion data, that at least in the leaves, there is no disappearance of auxin, except in growing tissue.

Table IV

Auxin Extracted with Ether from Various Portions of Developing Fronds and Rhizomes of Osmunda cinnamomea. Results Expressed as Plant Units * per Gm Fresh Wt of Tissue

Tissue Extracted	Plant units per gm **
Crozier plus pinnae	37.6
Zone of elongation	54.0
Upper zone of maturation	53.5
Lower zone of maturation	54.0
Leaf base	56.3
Rhizome directly below attachment of leaves	68.3
Rhizome 1.5 to 3 cm below attachment of leaves	82.0
Lower zone of maturation, leaves with all laminar tissue removed 48 hours before extraction	4.6
Rhizome from plant above, with all laminar tissue removed 48 hours before extraction	3.2
Lower zone of maturation, normal plant	53.8
Rhizome, normal plant	118.0

* Degrees curvature per Avena plant per gm tissue extracted.

** All values given were below maximum curvature for day.

Discussion

The enzymatic auxin-inactivating system obtained in water extracts of Osmunda leaves was described in a previous paper (1). In view of the possible morphogenetic implications in the activity and distribution of such a system, it becomes important to determine whether or not the system is active in vivo. Furthermore, it must be possible to construct a reasonable hypothesis about the system as controlling growth. Also, in vivo experiments on the activity of the system must correlate with in vitro experiments, if one is to use conclusions from the latter to suggest interpretations for the former. If the system is a growth-limiting factor, one would anticipate finding its activity to be highest in such tissues, in Osmunda, as the zone of maturation, leaf base, and rhizomes below the current year's leaves. These regions have completed their growth, and are no longer responsive to added or endogenous auxin. Tissues such as those of the rachis above the zone of elongation, which have not yet become responsive to added or endogenous auxin, might also show high activity. Rapidly grow-

ing tissues such as the zone of elongation might be expected to show lower activity.

However, none of the data obtained either from in vitro or in vivo experiments support the hypotheses listed above. Auxin destruction activity in leaf extracts is indeed high in the crozier, above the zone of elongation, but there is no disappearance of auxin within the rachis in vivo, in this region (see table I). This activity remains high in extracts of the zone of elongation, but rather than becoming higher in the zone of maturation, it is either considerably reduced, or completely absent. These results appear to be just contrary to those of Galston and Dalberg (4), which showed that extracts of the youngest tissues of etiolated pea seedling epicotyls contained the lowest IAA-oxidase activity, while extracts of progressively older tissues showed increasing activity. In Osmunda, however, one cannot establish a causal relationship between either initiation or cessation of elongation of the rachis and in vitro auxin inactivation from the data presented above.

Studies on the relative activity of the system in vitro, through the growing season (1), present an equally uncertain picture. If auxin inactivation is a growth-limiting factor of importance in rachis tissue either before or after elongation (or both), one might expect the activity of the system to be high in extracts of dormant fronds, or fronds just breaking dormancy, falling to a lower level as the fronds enter their period of most active growth, and rising again as growth tapers off and the fronds become mature. Again, in vitro experiments do not support this premise; activity of the system is low while fronds are dormant, rises early to a high peak as the fronds begin to elongate, and appears to fall off again as the fronds enter their most active growth stage (although this drop could be accounted for by a localization of enzyme activity in the more apical portions in extracts of whole leaves), remaining low as the fronds mature (1). A morphogenetic interpretation of these data is equally difficult to make. The situation must be far more complex than that hypothesized above.

Diffusion data shown also make it difficult to accept the hypothesis of enzymatic auxin inactivation as a morphogenetic factor in this material. As Steeves et al (9) have pointed out, although there are dangers involved in basing any broad interpretations upon auxin yields obtained by diffusion using as powerful a physiological agent as cyanide, it is impossible to obtain consistent yields of auxin by diffusion from any part of the plant when cyanide is not used. Auxin in fairly large quantities clearly passes from the crozier into the zone of elongation, and from there, through the lower tissues into the rhizome. Ether extractions show a high level of auxin in the rhizomes of normal plants with developing leaves, and that this auxin comes from the fronds is demonstrated by the fact that it disappears both from the lower rachis and from the rhizome when laminar tissues are removed 48 hours prior to extraction. Furthermore, it has also been possible to show that auxin can be obtained from the rhizome by diffusion, if this is done within approximately a millimeter below the attachment of the current year's leaves, on certain occasions. The pattern of auxin distribution in fronds of this fern, elucidated by the use of the cyanide technique, provides a reasonable picture which is unobtainable without the use of cyanide. A similar situation was found in shoots of Cercidophyllum, which also gave negligible and inconsistent yields of auxin without cyanide (12).

If one considers that KCN, applied to cut surfaces, poisons endogenous auxin destruction, one must then consider that it poisons it selectively, since neither auxin production by the pinnae, auxin utilization by the zone of elongation, auxin transport by any part of the leaf, nor polarity of auxin transport by the rachis appear to be affected by this treatment. Moreover, diffusion studies on two other ferns, *Adiantum pedatum* L. (maidenhair fern) (9) and *Pteretis pensylvanica* (Willd.) Fern. (ostrich fern), the latter having a growth habit closely comparable to that of *Osmunda cinnamomea*, have indicated that auxin yields from these species are unaffected by the cyanide treatment. It is concluded, therefore, that these ferns lack the inactivation system described for Osmunda, and that, in these plants, no other systems influencing auxin production, transport, and utilization are affected by cyanide, in the concentrations used.

Several authors (2, 4, 13) have suggested morphogenetic interpretations based on the distribution and activity of an auxin destruction system in their material. Other authors (5, 14, 15) have not attempted such interpretations. Their data give a picture of the distribution of the system which, like that described in the present paper, is difficult to reconcile with the concept of auxin destruction as a morphogenetic factor. (We must limit auxin destruction to auxin disappearance without accompanying growth.) Recently Platt (7) has concluded that an auxin destruction system found in extracts of normal and crown gall tissues of *Vitis vinifera* L., grown in sterile culture, is in all probability a cut surface and crushed tissue phenomenon only. Furthermore, although Tang and Bonner (11) have shown IAA-oxidase activity both in vivo and in vitro by epicotyls of etiolated pea seedlings, they were able to show only slight activity by an acetone precipitate of an extract of normal green epicotyls, and none at all either in vitro, by crude extracts, or in vivo, by green epicotyls. Galston and Baker (3) were able to show IAA-oxidase activity by extracts of green epicotyls, but only after acetone precipitation and double washing with acetone to remove a naturally occurring inhibitor (10). Thus, evidence from the literature does not give a convincing argument for a physiological IAA-oxidase system acting in vivo.

Evidence presented above shows a lack of correlation between auxin destruction in vitro and auxin disappearance in vivo, in Osmunda leaves. Tissues which transport auxin without destroying it, particu-

larly the crozier and zone of maturation, will inactivate added IAA in crude water extracts. Nevertheless, neither the diffusion data, particularly that obtained by using the cyanide technique, nor the extraction data, by themselves, are unequivocal. But ether extractions of rhizome tissue show that when the only source of endogenous auxin, the pinnae, are removed, extractable auxin disappears completely from the rhizome. Yet, a water extract of rhizome tissue consistently fails to inactivate added IAA. In this case, the situation is reversed from that in the zone of maturation, and auxin disappears in the living tissue, a water extract of which will not destroy it. Transport of auxin to lower parts of the rhizome cannot explain its disappearance in this tissue, for other experiments indicate that little or no auxin is actively transported by the rhizome more than a millimeter below the current year's leaves. The eventual fate of this auxin is unknown.

Although no one type of experiment by itself is absolutely conclusive, the total evidence presented and discussed above strongly suggests that enzymatic auxin destruction in vivo can play at most a negligible role in control of growth in the leaves of *Osmunda cinnamomea*, and is in all probability an artifact resulting from cutting or otherwise damaging tissues. Therefore, investigations of a different nature must be carried out to determine just what the controlling factors are.

Summary

In the fern *Osmunda cinnamomea* L., endogenous auxin is produced by the pinnae only, and both endogenous auxin and IAA applied in a lanolin-water emulsion move in a strictly polar fashion. Auxin diffusion data show that the only disappearance of endogenous auxin in the rachis is in the zone of active elongation. KCN, applied to cut surfaces throughout, to prevent cut surface auxin inactivation during diffusions, is effective less than 1 cm above the point of application in preventing inactivation, at least by damaged tissues.

The final rate of diffusion of IAA, applied in a lanolin-water emulsion to the epidermis, from sections of the zone of maturation is constant for sections of comparable age and diameter, and independent of the length of the transport path. Since as much auxin comes through long sections as short, it is suggested that there is no endogenous destruction within this tissue.

A comparison of in vivo auxin disappearance and in vitro auxin inactivation by the rachis shows that the crozier and zone of maturation will transport auxin without destroying it, while water extracts from the identical tissue, particularly the crozier, will inactivate IAA. The only auxin disappearing in vivo disappears in growing tissues. The concentration of ether extractable auxin, determined on a weight basis, increases from the crozier, bearing the pinnae, to the zone of elongation and tissues below. It does not diminish down the entire length of the rachis, but accumulates in the rhizome. There is no evidence for inactivation within the rachis, from these data.

Water extracts of rhizome tissue fail to destroy added IAA, but a disappearance of ether-extractable auxin is noted in this tissue following the removal of the auxin-producing fronds. The eventual fate of this auxin is unknown, but it is suggested that neither active transport nor the enzyme system under consideration are responsible for its disappearance.

The enzyme system is considered to be active only at cut surfaces and in tissue extracts, and to be of little importance in the normal growth physiology of this fern.

The authors wish to express their appreciation to Professor K. V. Thimann for his advice and encouragement during this investigation, to Dr. R. S. Platt for his suggestions and assistance during the later stages of the work, and to Professor Thimann, Dr. G. Morel, Dr. J. P. Nitsch and Dr. C. S. Yocum for their careful reviews of the manuscript.

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EFFECTS OF OXYGEN AND CARBON DIOXIDE LEVELS UPON ABSORPTION OF POTASSIUM BY PLANTS[1,2]

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Soil aeration has been recognized for years as an important factor in plant competition and crop production. Earlier work on the problem has been reviewed by Cannon and Free (6), Clements (8), Leather (18), and Parker (21), and the more recent literature by several writers (9, 10, 15, 20, 23, 27, 30). Most of the research on aeration suggests that plant roots are surprisingly tolerant of reduced O_2 and high CO_2 pressures. Short time experiments, however, might fail to show small differences in root growth or absorption rates which would be accumulative with time.

Interest in the problems of soil aeration has been increased in Iowa by the poor crop yields obtained in some areas with cultural programs which omit plowing during seedbed preparation (5, 17). Corn has shown serious potassium, and possibly other, deficiencies with listing and subsurface tillage on heavier glacial till soils (fig 1). Hoagland and Broyer (13) and others (3, 25, 26) have shown the importance of respiration and oxygen supply in certain types of mineral accumulation by plants. Chang and Loomis (7) and Vlamis and Davis (28, 29) have shown that high concentrations of CO_2 are injurious also, although the question of the relative importance of O_2 deficiency and CO_2 excess in field soils is not yet fully established (10, 12, 14, 19, 24, 30).

FIG. 1. Corn planted on plowed land, *left*, and hard listed, *right*. Potassium fertilizer largely offset the unfavorable effects of listing or other seed bed preparation methods which reduced gas exchange in soils.

The research reported here was undertaken to determine the relative effects of O_2 and CO_2 on potassium absorption by roots of maize plants growing in soil or in water culture. Soybeans were used in one experiment, and incidental data were collected on the effect of soil gases on transpiration rates of both corn and soybeans. However, the emphasis has been on the absorption of potassium by corn because of the striking potassium deficiencies obtained with corn in the field cultivation plots and the sensitivity of potassium absorption to poor aeration as shown in the work of Chang and Loomis (7).

MATERIALS AND METHODS

APPARATUS: Special equipment was designed to discharge various gas mixtures through glazed, one-gallon pots of soil or quart jars of nutrient solution. The mixing of the gases and the control of the flow rate were accomplished by the use of calibrated glass capillaries as described by Bartholomew and Broadbent (4). Gas from tanks of O_2, N_2 and CO_2 was used to obtain the desired mixtures. Figure 2 illustrates the system of mixing and delivery of gases to the bottom of a one-gallon pot. Gas was delivered to the pot at a pressure of 100 cm of water. A differential pressure of 100 cm of water forced the gases through calibrated capillaries to the mixing chamber. In practice it was only necessary to adjust the needle valves at the gas source so that a slight excess of gas escaped through the water pressure towers. By careful adjustment of the water pressure, gases of the desired composition were readily obtained. Figure 3 shows diagrammatically an arrangement of the above type of apparatus for delivery of 6 different mixtures

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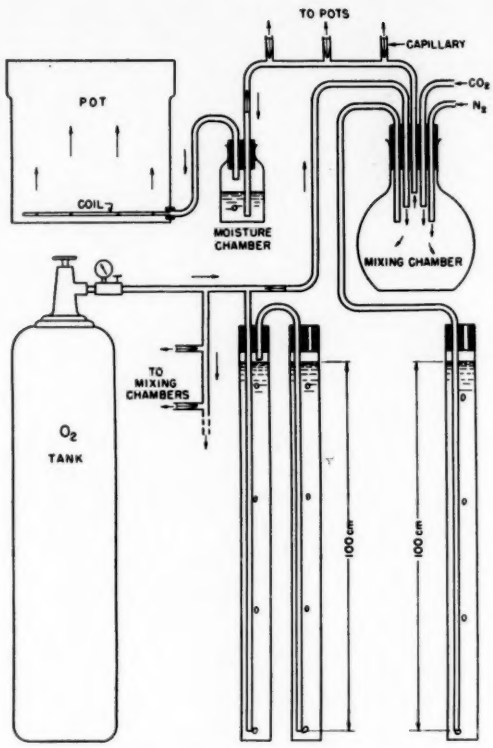

FIG. 2. Diagram of method used to obtain gas mixtures. The capillary inserts were calibrated to serve as flow meters. Tank valves were opened to waste some gas through the water-filled bypasses.

of gases. A single pressure tower connected between the 6 mixing chambers and the capillaries distributing the gases to the pots was found to be satisfactory. A minimum of neoprene rubber tubing, placed only at connection points between Pyrex glass tubing, was used in the apparatus.

In the soil aeration experiments, the rate of flow of gases was adjusted to 10 l per pot per day; in the nutrient culture experiments, gas flow was 20 l per day. The accuracy with which the gases were metered is indicated by the data of table I.

SOIL AERATION EXPERIMENTS: Two soil aeration experiments were conducted in which corn and soybeans were used as indicator plants. A mixture of equal parts of Carrington loam soil and builder's sand was placed in the pots and maintained near field capacity by frequent weighing and rewatering of the pots. Fertilizer applications were made to all pots at the rate of 100 lbs of N per acre as ammonium nitrate, 200 lbs of P_2O_5 per acre as calcium dihydrogen phosphate, and 50 lbs of magnesium sulfate per acre. All fertilizer applications were made on the basis of weight of soil per pot and 2×10^6 lb soil per acre. The rates used were known to be optimum for these soils.

Experiment 1, using corn, tested the effect of varying percentages of O_2 and CO_2 on the growth of corn over a 4-week period. Pots were set up in a split-plot design with 4 replicates, in which the main treatments were no K and 100 lbs K per acre as KCl. Four aeration treatments, begun as soon as the plants emerged and continued throughout the experiment, made up the sub-plots. Four hundred grams of white quartz flour was applied to the soil surface of each artificially aerated pot in an attempt to impede the diffusion of gases. The soil atmosphere was sampled through small copper tubes placed in the soil at the beginning of the experiment, and analyses were made with a Haldane gas analyzer. Dry weights of top and roots of plants were recorded.

In experiment 2, both corn and soybeans were grown in pots as in experiment 1. However, the experimental material was 4 weeks old before any aeration or addition of potassium was carried out. Nitrogen and P were supplied at planting. The aeration experiment was begun by covering the corn pots carefully with plastic film and the soybean pots with a vaseline-paraffin mixture. Then followed in order the initiation of aeration, the addition of K (150 lbs/acre) 24 hours later, the harvesting of roots and tops after an additional 48 hours of aeration, and the analysis of the total plants for K by the dipicrylamine method of Lawton (16). The K nutrient solution was applied through the covers with a large hypodermic syringe. Water to maintain the soil at field capacity was added

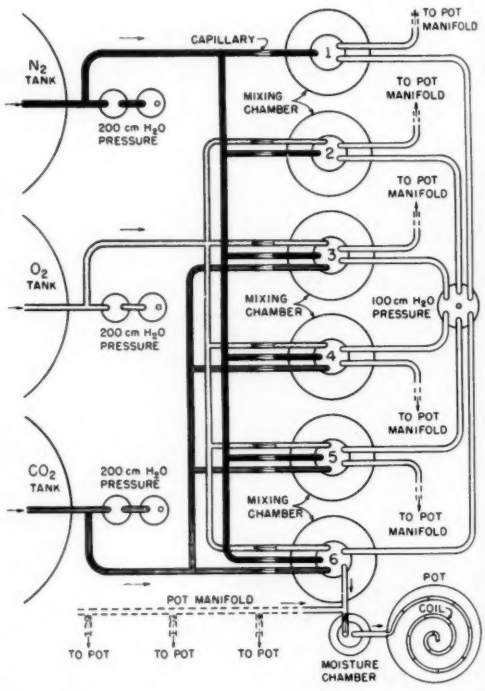

FIG. 3. Diagram of piping and flowmeter system for use with 6 gas mixtures.

TABLE I

EXPECTED AND ACTUAL COMPOSITION OF AERATING GASES

EXPECTED		ACTUAL *	
O_2	CO_2	O_2	CO_2
%	%	%	%
0	0	0.45 **	0
20	0	20.69	0
0	5	0.21	4.80
0	10	0.33	10.47
0	20	0.10	20.30
0	50	0	48.50

* Gas samples taken at the inlet to the jars.
** Impurity in N_2 gas.

in the same manner and recorded. Gas samples from the soil were obtained for analysis by means of a 10 ml syringe and a 4-inch hypodermic needle. The gas sample was drawn into the syringe and then discharged into the burette of a Haldane gas analyzer through a mercury-filled rubber sidearm tube. This technique of gas analysis was used throughout subsequent experiments. The Pauling (22) oxygen analyzer proved unsatisfactory for measurements involving the small gas samples.

NUTRIENT SOLUTION AERATION EXPERIMENTS: In these experiments corn was grown in Hoagland's (13) solution for 2 to 4 weeks and was used as the indicator plant to study the effect of various O_2 and CO_2 levels upon water and potassium absorption. The corn plants were supported in quart Mason jars by squares of paraffin-treated plywood. During the period of aeration the plywood square was sealed to the jar with plasticene, and only small cotton-plugged openings were left immediately surrounding the plants. The jars were wrapped in black paper to prevent the growth of algae in the nutrient solution. Solutions were changed at approximately 5-day intervals, depending upon the age of the plants. Phosphorus and iron were supplied alternately in the nutrient solution (11), the iron being added as a 0.4 % solution of ferric tartrate.

Duplicate gas treatments were used, with the gas discharged in a stream of small bubbles from two glass capillary tube outlets in the bottom of the jar. The water lost through transpiration and evaporation was recorded and replenished by frequent additions of distilled water. Samples of the nutrient solution were obtained periodically for K analysis with the Perkin-Elmer Model-18 flame photometer (2). Frequent analyses were made on the entering gases, and in one series we measured the dissolved O_2 content of distilled water, substituted in the experiment for the nutrient solution. Moderate amounts of O_2 were often present in the nitrogen tanks, and in some experiments this was removed by passing the N_2 through towers of alkaline pyrogallol.

Most of these experiments were designed to test the absorption of K and water from solutions bubbled continuously with varying gas mixtures. In general the plants were grown for 3 weeks in unaerated cultures, then run on test for 5 days. In one experiment, aeration treatments were started at 2 weeks and continued for 3 weeks to observe accumulative effects of growth as well as of K absorption. Transpiration was measured as water loss from the containers. Treatment effects were assumed to have been more closely related to water absorption than to effects on the leaves. Potassium absorption was determined by periodic analyses of the culture solutions.

Approved experimental designs were used throughout, and all data were analyzed by appropriate methods; usually by analysis of variance. All statements of significance are based on such analyses, and indicate 0.05 or 0.01 levels of probability.

EXPERIMENTAL RESULTS

EXPERIMENT 1—CORN IN SOIL: These plants were grown in a mixture of equal parts of soil and sand. All pots received N, P, Ca, Mg and S fertilizer and half received K at the rate of 100 lbs per acre. Gas treatments, designed to give wide differences in soil aeration, were started as soon as the plants emerged and continued throughout the experiment at the rate of 10 l/day. The pots were covered only with a layer of rock flour, and there was a rapid gas exchange with the atmosphere through this sandy soil (table II). The mixture containing 40 % O_2 gave the best yields of tops, but no treatment was significantly better than

TABLE II

COMPOSITION OF THE SOIL ATMOSPHERE AND YIELDS OF CORN GROWN IN SOIL AT 2 K LEVELS AS AFFECTED BY AERATION DURING GROWTH WITH VARIOUS GAS MIXTURES

COMPOSITION OF GASES		DRY WEIGHT			
		TOPS		ROOTS	
AERATING GAS	SOIL ATMOSPHERE, 6" DEPTH	NO K	100 LBS K/ACRE	NO K	100 LBS K/ACRE
		gm	*gm*	*gm*	*gm*
100 % N_2	16.4 % O_2, 0 % CO_2	5.96	9.04	2.57	5.87
100 % CO_2	10.82 % O_2, 30.05 % CO_2	*2.55*	8.94	*0.88*	*2.63*
40 % O_2, 60 % N_2	25.73 % O_2, 0.20 % CO_2	5.33	9.16	2.82	6.06
No aeration	21.3 % O_2, 0.25 % CO_2	5.36	8.43	2.64	6.15
Average	.....	4.80	8.89	2.23	5.18

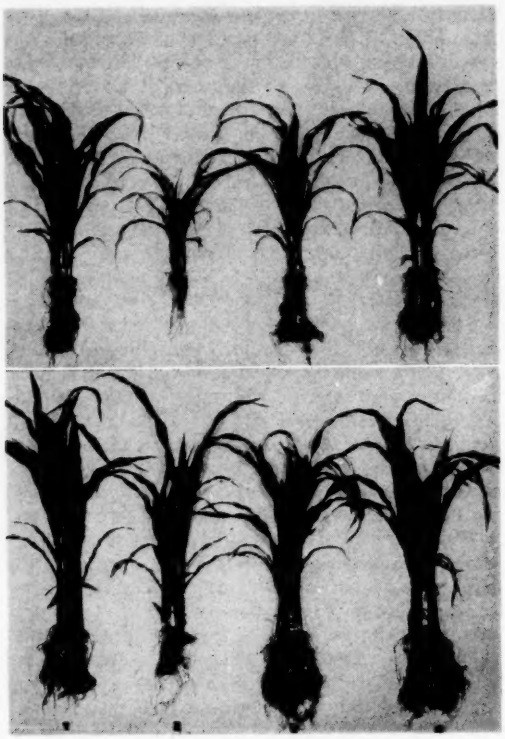

FIG. 4. Corn grown in soil with varying aeration and K levels. Left to right: aerated with N_2, CO_2, 40 % O_2, no aeration. *Above*, −K; *below*, +K.

the control. Analyses of the soil air showed that O_2 was reaching all pots in liberal quantities. Where the soil air contained 30 % CO_2 the plants showed a marked reduction in root growth (fig 4, table II), particularly with low K. Top growth was reduced nearly 60 % by the CO_2 treatment in the absence of K, but not at all with K addition. These results suggest that high CO_2 may have the same depressing effect on K absorption in soil that was found in solution cultures by Chang and Loomis (7), although they are confounded by the poor root growth in the presence of high CO_2. Relative root development in the high CO_2 cultures was about half that in the controls, and was not improved by addition of K. At the same time the addition of K increased total growth in the presence of CO_2 nearly three times as much as in the controls, so that a combined effect of reduced root growth and reduced absorption seems probable.

EXPERIMENT 2—CORN AND SOYBEANS IN SOIL: This experiment was similar to the previous one except that the plants were grown for 4 weeks without K addition or aeration treatments to eliminate root-growth effects. Pots were then sealed with sheet plastic or vaseline-paraffin mixture and gas treatments started at the rate of 10 l/day. Potassium was added to the treated pots on the second day at the rate of 150 lbs/acre, and aeration was continued for another 48 hours. The plants were then harvested, dried and analyzed for K. Gain in dry matter and potassium contents and water losses of the two species are shown in table III. The seals used were more effective than the rock flour, and good agreement was obtained between aeration mixtures and soil gas analyses. Reducing O_2 to 4 % had no effect on the absorption of K or water by plants. Added CO_2 was without effect in the presence of normal O_2 until it reached a concentration of about 20 %. At this point K absorption was reduced, and at 50 % CO_2 both K and water absorption were reduced significantly.

EXPERIMENT 3 AND 4—CORN IN SOLUTION CULTURE: Experiment 3 was a repetition of Experiment 2 except that the plants were grown in solution culture instead of soil and aerated during a 5-day test period at the rate of 20 l per quart jar per day. The data, presented graphically in figure 5, indicate again that 10 % CO_2 was not injurious in the presence of 20 % O_2 but that higher percentages reduced K absorption.

TABLE III

DRY WEIGHT AND K GAINS, AND WATER LOSSES OF CORN AND SOYBEANS GROWN IN A SOIL-SAND MIXTURE AND VARIOUSLY TREATED WITH GAS MIXTURES

AERATION TREATMENT *	K FERT., LBS/ACRE **	SOIL GAS ANALYSIS		AVE GAIN IN DRY WT/POT		AVE GAIN IN K CONTENT/POT		AVE WATER LOSS/POT	
		O_2	CO_2	CORN	SOYBEANS	CORN	SOYBEANS	CORN	SOYBEANS
		%	%	*gm*	*gm*	*mg*	*mg*	*gm*	*gm*
100 % N_2	150	4	2	6.12	3.44	57.6	29.6	659	945
20 % O_2	150	17	4	5.23	3.01	52.9	35.1	603	912
20 % O_2, 5 % CO_2	150	18	7	5.81	... †	53.2	...	616	800
20 % O_2, 10 % CO_2	150	18	11	4.49	3.05	59.2	35.6	616	952
20 % O_2, 20 % CO_2	150	18	18	5.05	3.19	40.0	26.3	625	922
20 % O_2, 50 % CO_2	150	18	45	6.08	2.35	27.8	9.2	497	649
No aeration	None	19	2	5.35	2.47	7.3	14.7	...	...

* Nitrogen gas made up the remaining percentage.
** Potassium fertilizer was applied 24 hours after the aeration was started.
† Results invalidated by disease.

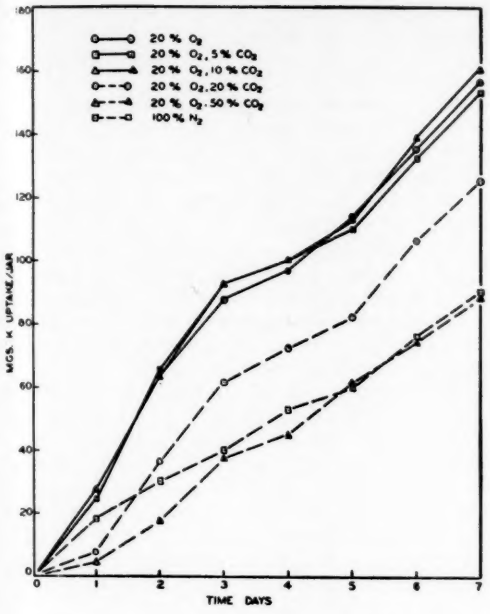

FIG. 5. Absorption of K from solutions bubbled continuously with the indicated gas mixtures. Low O_2 in commercial N_2 or 20 % or more CO_2 reduced K absorption significantly.

Water absorption, also, was reduced by the high CO_2, with differences significant beyond the 1 % level. Bubbling with commercial N_2 reduced absorption of K but not of water.

In experiment 4 the gas mixtures were changed to show the effects of CO_2 in the presence of the approximately 0.5 % O_2 impurity in commercial N_2 gas. Whereas 20 % of CO_2 was required to produce significant reductions of K absorption in the presence of 20 % O_2, 5 % CO_2 gave highly significant reduction when O_2 was added only as an impurity in the N_2 (fig 6). Sweeping these closed jars with N_2 increased K and water absorption over that of the control. These results recall those of Vlamis and Davis (28) who found that bubbling rice cultures with methane reduced the CO_2 content of the cultures and increased plant growth.

EXPERIMENT 5: This experiment was designed to obtain further evidence of the detrimental effects of CO_2 upon K and water absorption when small amounts of O_2 are available. The results shown in table IV indicate that the presence of 10 % CO_2 markedly reduced the effectiveness of 0.5 and 1 % O_2. Interaction effects are obvious in the data. Aerating with 5 % O_2 and 95 % N_2 doubled K absorption above the unaerated control. Adding 10 % CO_2 with 5 % O_2 gave a significant reduction in K absorption, but left this value high above that of the control. With 0.5 % O_2, 10 % CO_2 reduced K absorption by 52 %, but with 5.0 % O_2 the corresponding reduction was only 20 %. In contrast to the results with K, absorption of water was not reduced significantly more by 0.5 % O_2 + 10 % CO_2 than by the low O_2 alone.

TABLE IV

POTASSIUM AND WATER ABSORPTION BY CORN PLANTS IN HOAGLAND'S NUTRIENT SOLUTION WITH VARYING O_2 AND CO_2 LEVELS

AERATION TREATMENT *	ACTUAL COMPOSITION OF AERATING GASES		AVERAGE ABSORPTION	
	O_2	CO_2	K	H_2O
	%	%	*mg*	*gm*
0.5 % O_2	0.52	0	33.1	132
1.0 % O_2	1.04	0	34.5	167
5.0 % O_2	5.19	0	75.6	178
0.5 % O_2, 10 % CO_2	0.44	10.22	16.0	127
1.0 % O_2, 10 % CO_2	1.01	10.35	19.2	136
5.0 % O_2, 10 % CO_2	5.06	10.13	60.9	183
No aeration	....	....	38.5	154

* Nitrogen gas makes up the remaining percentages.

EXPERIMENT 6: In this series corn was grown in Hoagland's solution with constant aeration after the plants were established. The data in table V indicate the good growth which may be obtained with considerable accumulations of CO_2, provided O_2 is not critically low.

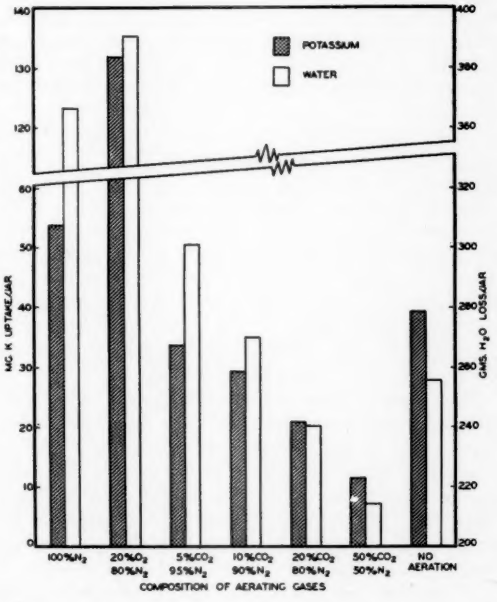

FIG. 6. Absorption of K and water from solutions bubbled with the indicated gas mixtures. Lower concentrations of CO_2 were effective in reducing absorption when O_2 was limited to that present as an impurity in the N_2 gas.

TABLE V

YIELDS OF CORN GROWN 3 WEEKS IN HOAGLAND'S NUTRIENT SOLUTION AERATED WITH GASES OF VARYING COMPOSITION

Composition of aerating gas *		Ave dry wt of plants	Dissolved O_2	
			Without plants	With plants
O_2	CO_2			
%	%	*gm*	*ppm*	*ppm*
0	0	5.74	0.45	0.04
20.10	0	9.16	5.96	1.03
14.75	4.72	7.46	4.43	0.49
9.71	9.66	7.06	3.31	0.18
5.31	16.12	7.24	1.61	0.09
0	20.53	3.46	0.13	...
No aeration		5.52	6.58	0.23

* Nitrogen gas makes up the remaining percentages.

The O_2 analyses were made on parallel cultures with uniformly grown plants to eliminate size of plant effects. The data indicate the important effect of moderate size plants on the O_2 content of solution cultures and, at the same time, show the low levels of free O_2 with which reasonably satisfactory growth is possible.

DISCUSSION

The results of our experiments show clearly that CO_2 may reduce both root growth and the rate of K and water absorption by unit root surfaces. The levels required are high, however; something like 5 % CO_2 in equilibrium with the soil moisture when O_2 is less than 1 %, and 20 % CO_2 with 20 % O_2. Such gas levels are rarely reported in the usual analyses of soil gases.

The point may be emphasized by field results. Analyses in the plots pictured in figure 1 showed about 1 % of CO_2 and 19 % of O_2 in the soil gases, with no differences due to treatment. When a fertilizer containing 12 % K_2O was used, the CO_2 content of the average soil gas obtained in a bulk sample was nearly doubled, but the poor growth shown by the listed plants in figure 1 was corrected. The indication is that an interference with K absorption, of the type shown in figure 4, was present in the field, although CO_2 percentages were 20 times lower.

Soils which show potassium deficiency symptoms when apparently well supplied with this element, follow a fairly definite pattern. They are heavy, silts to silty clays; they have a poor structure; they are compacted by weather or by treatments; and they are relatively high in calcium, frequently with $CaCO_3$ concretions in the sub-soil. The difficulty may be accentuated by high soil moisture or low temperature.

Ostensibly the problem is one of aeration. The objection that analyses of soil gases do not show the O_2 and CO_2 percentages which have been demonstrated to reduce K absorption can be met by assuming that micro-aeration, in the immediate root zone, may be poor, and that this localized depletion of O_2 and accumulation of CO_2 cannot be measured by gross analyses of the soil gases. This assumption explains why the difficulty is not encountered in soils of coarser texture.

The experiments reported here have been aimed at demonstrating significant reductions of K absorption in short-time tests. In the field we might expect that conditions which reduced K absorption by only a few percent would also reduce root growth. This reduced root growth would compound the K deficiency effect and thus lead to the poor growth and yields observed in certain fields. Perhaps the antagonistic effect of calcium on K absorption (1) is a major factor here, and is an explanation of why soil structure does not appear to be so critical farther east, or even on the more leached soils of eastern Iowa.

We suggest that the K deficiency observed in certain Iowa fields which appear to be adequately supplied with this element is due to a combination of calcium antagonism and slow rates of gas exchange, so that the micro-aeration of the absorbing roots drops below the level required for effective absorption of K. The work of Chang and Loomis (7) indicates that the absorption of K will be reduced more quickly under these conditions than that of other elements.

SUMMARY

The absorption of potassium by corn roots as well as the growth of the roots may be markedly reduced by low supplies of O_2 or high concentrations of CO_2. Oxygen pressures obtained by bubbling culture solutions with 1 % O_2 in N_2 gave highly significant reductions in the absorption of K in short time experiments. Reducing O_2 to 5 % had little or no effect.

The effect of added CO_2 varied with the partial pressure of O_2. With O_2 at 1 %, 5 % of CO_2 gave approximately the same reduced K absorption as was obtained with 20 % CO_2 in the presence of 20 % O_2.

The accumulative effects of reduced K absorption and reduced root growth may result in near failure of plant development, even though the soil or solution medium is adequately supplied with K by usual criteria.

The degree of depletion of O_2 or of accumulation of CO_2 necessary to reduce K absorption in a sandy soil or a solution culture is much greater than that measured by gross analyses of soil gases in fields showing similar symptoms. We postulate that the micro-aeration of the soil in immediate contact with the absorbing roots is not measured by gross analyses, and that this aeration, augmented by an antagonistic action of high calcium levels on K absorption, explains the frequently severe symptoms of K deficiency observed on Iowa soils which are apparently adequately supplied with K.

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INHIBITION OF EXCISED TOMATO ROOTS BY SULFANILAMIDE AND ITS REVERSAL BY P-AMINOBENZOIC ACID AND N-PTEROYLGLUTAMIC ACID[1]

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Studies with antimetabolites (structural analogs biologically antagonistic to metabolites) have yielded a considerable amount of new information concerning metabolic pathways and functions of known metabolites in animals and microorganisms. The principles and possibilities are presented in monographs by Wooley (26) and Martin (11). Reference may also be made to the discussions by Shive (17, 18, 25) in

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which the technique of "Inhibition Analysis" is described.

The work described here deals specifically with sulfanilamide. It represents part of a program designed to determine the value of the excised tomato root as a biological system to which antimetabolites may be applied with the object of revealing possible metabolic interrelationships and the effects of known metabolites on the morphological development of the root system.

Inhibition of growth of a higher plant (garden cress) by sulfanilamide was shown by Fourneau et al (6). The experimental reversal of sulfonamide inhibition by *p*-aminobenzoic acid (PABA), using seeds or seedlings, has been demonstrated (5, 7, 9, 10, 14, 20, 24). Three papers are known (1, 4, 5) which report inhibition of growth of excised roots (grown in sterile culture) by sulfonamides and reversal of the inhibition by PABA or some other agent. In one of these papers (4) an apparent competitive antagonism, as determined by growth of the main axis, was observed between PABA and sulfathiazole.

Experiments reported here were designed to determine 1) whether sulfanilamide would inhibit growth of excised tomato roots, 2) whether any inhibition could be antagonized by PABA, n-pteroylglutamic acid (PGA), methionine, purines, and pyrimidines, and 3) whether it was possible to establish that any antagonism was competitive or non-competitive.

Materials and Methods

The general techniques are described elsewhere (2). The basal medium was that of White (23) except that the sucrose concentration was 1.5% and glycine and niacin were omitted. Within any one experiment, initial pH's of media were adjusted, where necessary, so that the pH of each treatment was approximately the same and within the range 4.8 to 5.0. The clone of excised tomato roots used as a source of inocula was that designated as R5 (3). Cultures were harvested and measured on the sixth day after inoculation.

All substances, other than the PABA, tested for effects on inhibition by sulfanilamide were purchased from Nutritional Biochemicals, Inc. The PABA was purchased from Eastman Kodak Company and the sulfanilamide from Delta Chemical Works. Unless stated otherwise, all substances included in any one medium were autoclaved in that medium. Throughout the description of results, concentrations are referred to as times a given standard concentration. The standard concentration 1.0 was equal to 4×10^{-7} M of the free compound.

Results

The inhibitory effects of either sulfanilamide, PABA, or n-pteroylglutamic acid (PGA) added to the basal medium are shown in figures 1 and 2. Each treatment consisted of at least nine replicates. Inhibition resulted with lower concentrations of PABA than either sulfanilamide or PGA. In other experiments inhibition by sulfanilamide was complete at a concentration equal to or less than 10,000 standard but greater than 5,000 standard. Inhibition of growth of laterals occurred at lower concentrations of sulfanilamide than were required to inhibit growth of the main axis. The converse of this was true in the case of PABA and PGA. With these substances the inhibition in growth of main axis was correlated with an increase in growth of laterals. This effect was observed with PABA in the experiment illustrated in figure 1 and with PGA in five other experiments (table I) in addition to that illustrated in figure 1.

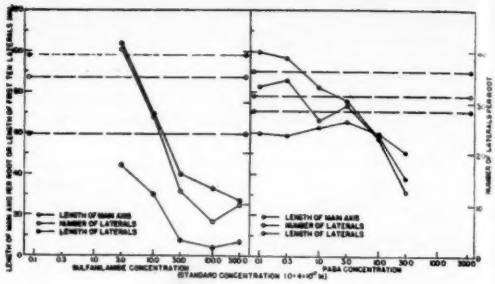


FIG. 1. The effect of concentration of either sulfanilamide or *p*-aminobenzoic acid (PABA). The horizontal broken lines show the level of growth in the control medium. The term "first 10 laterals" ("10 basal laterals" in tables) refers to the 10 basal laterals nearest the cut end of the root.

Stimulation of growth in length of the main axis, and in some cases of laterals, was observed with both sulfanilamide and PABA (fig 1 and table II). Results with PGA were very variable, and stimulation in growth of main axis was observed in only one experi-

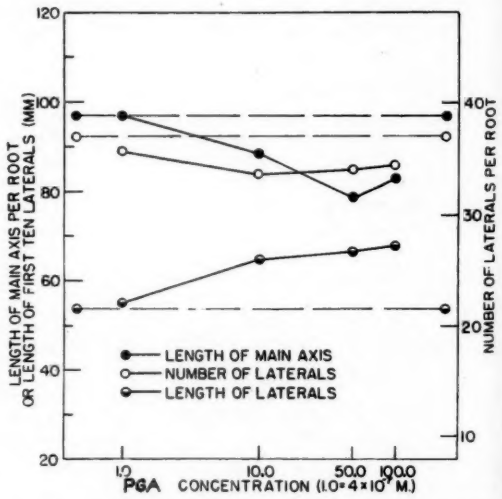

LENGTH OF MAIN AXIS PER ROOT OR LENGTH OF FIRST TEN LATERALS (MM)
NUMBER OF LATERALS PER ROOT
LENGTH OF MAIN AXIS
NUMBER OF LATERALS
LENGTH OF LATERALS
PGA CONCENTRATION (1.0=4×10⁻⁷ M.)

FIG. 2. The effect of concentration of n-pteroylglutamic acid (PGA). The horizontal broken lines show the level of growth in the control medium.

TABLE I

STIMULATION OF GROWTH OF LATERALS ON SLIGHT INHIBITION OF GROWTH OF MAIN AXIS BY PGA IN BASAL MEDIUM

EXPT NO.	CONTROL		CONC OF PGA †	CONTROL + PGA	
	FINAL LENGTH OF MAIN AXIS PER ROOT	TOTAL LENGTH OF 10 BASAL LATERALS PER ROOT		FINAL LENGTH OF MAIN AXIS PER ROOT	TOTAL LENGTH OF 10 BASAL LATERALS PER ROOT
	mm	*mm*		*mm*	*mm*
1	91.0 ± 4.49 * (9) **	73.4 ± 4.93	10.0	83.0 ± 5.36 (9)	82.6 ± 3.40
2	97.0 ± 7.12 (9)	54.3 ± 6.49	10.0	88.4 ± 6.05 (9)	65.2 ± 5.44
			100.0	83.4 ± 5.61 (9)	68.2 ± 3.96
3	107.2 ± 4.74 (9)	69.4 ± 6.65	10.0	89.8 ± 4.52 (9)	73.2 ± 6.49
4	111.6 ± 2.51 (8)	64.1 ± 6.99	10.0	100.8 ± 4.73 (9)	78.9 ± 6.42
5	119.9 ± 3.91 (8)	50.0 ± 3.53	10.0	114.8 ± 2.90 (9)	58.5 ± 2.45

* Standard Error = $\sqrt{\frac{\text{variance}}{\text{number of replicates}}}$ (Snedecor (19)).

** Number of replicates.

† $1.0 = 4 \times 10^{-7}$ M.

ment out of six. The concentration of PGA was 0.03 of the standard. This concentration was not used in the other five experiments, although concentrations close to, but above or below, 0.03 of the standard were used. Stimulation of growth of laterals by PGA was observed on a number of occasions. Stimulation in growth of the main axis with either sulfanilamide, PABA, or PGA was not necessarily accompanied by stimulation in growth of laterals. Indeed, the converse was true in some instances.

Sulfanilamide at concentration 100.0 × standard gave approximately 80 % inhibition in growth of main axis and 90 % inhibition in growth of laterals. The inhibition was completely removed on supplying PABA at 1.0 × standard (3.0 was slightly inhibitory) or by PGA at 30.0 × standard (100.0 increased the inhibition of main axis but stimulated growth of laterals). Because this high degree of inhibition could be overcome by PABA or by PGA it was possible to design experiments to test the effect of PABA or PGA at a series of inhibitory concentrations of sulfanilamide. The results of two such experiments are presented in tables IV and V. The range of inhibitory concentrations of sulfanilamide which could be examined was, however, limited because of the relatively low concentrations at which PABA or PGA proved inhibitory.

During the preliminary experiments on the effect of PGA in the presence and absence of sulfanilamide it was observed that, at high concentrations of PGA, there was a marked drift in pH and loss of color on autoclaving. This indicated destruction of the PGA. In order to ascertain whether such destruction would prejudice the results at the concentrations of PGA used in the experiments presented in table V, solutions of PGA were filtered through sterile Pyrex sintered glass filters and added to the autoclaved medium with necessary adjustments for volume. The

TABLE II

STIMULATION OF GROWTH ON ADDITION OF NON-INHIBITORY CONCENTRATIONS OF SULFANILAMIDE OR PABA TO BASAL MEDIUM

ADDITION	EXPT NO.	FINAL LENGTH OF MAIN AXIS PER ROOT	TOTAL LENGTH OF 10 BASAL LATERALS PER ROOT	CONC OF ADDITION *	FINAL LENGTH OF MAIN AXIS PER ROOT	TOTAL LENGTH OF 10 BASAL LATERALS PER ROOT
		mm	*mm*		*mm*	*mm*
Sulfanilamide	1	89.3 ± 4.29 (8)	62.3 ± 2.75	0.1	99.8 ± 5.02 (8)	65.6 ± 5.25
				1.0	108.3 ± 3.64 (8)	73.9 ± 4.73
				3.0	114.8 ± 6.04 (7)	54.3 ± 3.43
	2	97.7 ± 5.14 (7)	59.9 ± 3.20	3.0	103.9 ± 5.85 (8)	44.5 ± 3.43
PABA	1	89.3 ± 4.29 (8)	62.3 ± 2.75	0.001	104.1 ± 5.85 (8)	70.6 ± 6.37
				0.003	102.0 ± 5.50 (8)	68.0 ± 6.93
	2	89.9 ± 3.97 (9)	71.2 ± 4.09	0.1	100.0 ± 5.88 (9)	60.1 ± 6.55
	3	91.7 ± 5.85 (9)	79.6 ± 6.75	0.0001	103.8 ± 4.15 (9)	67.4 ± 2.55
				0.001	115.8 ± 5.47 (9)	69.0 ± 5.52
				0.01	100.1 ± 2.56 (7)	85.1 ± 6.60

* $1.0 = 4 \times 10^{-7}$ M.

TABLE III

EFFECT OF PGA STERILIZED BY FILTRATION (F) AND AUTOCLAVED (A) ON INHIBITION BY SULFANILAMIDE

CONC OF SULFANILAMIDE *	CONC OF PGA *	FINAL LENGTH OF MAIN AXIS PER ROOT	TOTAL LENGTH OF 10 BASAL LATERALS PER ROOT
		mm	*mm*
0	0	104.3 ± 5.43 (8)	63.0 ± 8.70
10	0	34.3 ± 4.42 (8)	.....
10	1.0 A	56.2 ± 2.24 (9)	16.5 ± 2.42
10	3.0 A	91.2 ± 4.08 (7)	54.1 ± 7.85
10	10.0 A	97.2 ± 2.18 (9)	47.6 ± 4.57
10	30.0 A	97.5 ± 2.01 (8)	47.2 ± 5.04
10	1.0 F	56.7 ± 3.92 (9)	20.7 ± 3.09
10	3.0 F	90.6 ± 5.53 (8)	48.1 ± 7.92
10	10.0 F	98.1 ± 2.64 (9)	55.8 ± 4.87
10	30.0 F	95.2 ± 3.64 (9)	61.4 ± 3.60

* $1.0 = 4 \times 10^{-7}$ M.

experiment was carried out on two occasions and gave similar results on each occasion. The results of one of these experiments are presented in table III. The data show that detrimental effects due to autoclaving PGA in the medium only occur at concentrations greater than those used in the experiment reported in table V.

The data in table IV show that the PABA itself was not inhibitory at any of the concentrations used. Other experiments, in addition to the control media in table IV, showed that inhibition due to sulfanilamide at 100.0 × standard could be relieved by PABA. Thus, the level of growth in any of the treatments shown in table IV was not a consequence of irreversible toxicity of either the sulfanilamide or PABA. Therefore, comparisons to determine the relationship between growth and ratio of analog to metabolite are valid. A non-competitive antagonism would be indicated if the level of growth, at a given concentration of metabolite and inhibitory analog, was not decreased by further increase in the concentration of the analog; a competitive inhibition would be indicated if the level of growth was constant with a constant ratio of analog to metabolite over a range of concentrations. Various factors may influence the expression of a competitive inhibition, and the lack of a strict relationship between level of growth and ratio of analog to metabolite does not necessarily mean that an antagonism is not competitive (see Wooley (19), pages 68–73).

In the case of PABA (table IV) the level of growth was not governed strictly by the ratio of analog to metabolite. However, the concentration of PABA required to overcome the inhibition by sulfanilamide increased with increase in the concentration of sulfanilamide. This was shown by both main apex and lateral roots. It is considered that the data indicate a competitive antagonism. In the case of PGA (table V) the results are variable but do show that the level of growth was controlled by the ratio of analog to metabolite. Thus there is a strong indication of competitive antagonism between PGA and sulfanilamide. The data also show that the inhibitory effect of PGA at 10.0 × standard (see table I) was relieved by sulfanilamide. This effect was not observed with PABA in any experiment.

TABLE IV

EFFECT OF PABA ON INHIBITION BY SULFANILAMIDE

CONC OF SULFANILAMIDE *	CONC OF PABA *	CONC RATIO	FINAL LENGTH OF MAIN AXIS PER ROOT	NO. OF LATERALS PER ROOT	TOTAL LENGTH OF 10 BASAL LATERALS PER ROOT
			mm		*mm*
0	0	...	110.8 ± 10.28 (5)	48.4 ± 3.93	66.0 ± 5.32
0	0.05	...	109.1 ± 4.31 (9)	40.0 ± 2.61	71.4 ± 3.53
0	0.1	...	107.7 ± 3.96 (7)	41.6 ± 3.00	70.7 ± 3.96
0	0.2	...	105.3 ± 5.99 (7)	43.7 ± 2.55	78.0 ± 6.14
0	0.3	...	105.7 ± 3.94 (9)	41.1 ± 2.12	68.3 ± 5.02
0	0.6	...	109.1 ± 4.36 (8)	46.3 ± 2.98	73.8 ± 5.16
100	0.6	166 : 1	109.0 ± 4.95 (7)	46.4 ± 3.81	44.4 ± 4.99
25	0.1		123.5 ± 8.81 (6)	52.7 ± 4.87	41.3 ± 2.03
50	0.2	250 : 1	109.9 ± 6.53 (7)	46.9 ± 3.91	28.6 ± 2.95
100	0.3		80.0 ± 5.27 (8)	38.0 ± 2.90	25.4 ± 3.62
25	0.05		93.6 ± 6.25 (9)	40.2 ± 2.47	31.4 ± 3.36
50	0.1	500 : 1	88.4 ± 8.30 (8)	35.1 ± 1.95	17.6 ± 1.57
100	0.2		75.9 ± 5.81 (8)	28.8 ± 2.66	12.5 ± 1.33
50	0.05	1000 : 1	79.0 ± 2.55 (9)	30.7 ± 3.14	10.8 ± 1.48
100	0.1		59.8 ± 4.64 (9)	14.9 ± 1.04	7.4 ± 1.25
25	0	...	72.4 ± 5.56 (8)	24.3 ± 2.49	16.0 ± 1.84
50	0	...	53.4 ± 1.29 (7)	12.6 ± 0.71	9.4 ± 0.64
100	0	...	43.7 ± 2.61 (9)	9.0 ± 0.63	4.8 ± 0.45

* $1.0 = 4 \times 10^{-7}$ M.

TABLE V

EFFECT OF PGA ON INHIBITION BY SULFANILAMIDE

CONC OF SULFANILAMIDE *	CONC OF PGA *	CONC RATIO	FINAL LENGTH OF MAIN AXIS PER ROOT	NO. OF LATERALS PER ROOT	TOTAL LENGTH OF 10 BASAL LATERALS PER ROOT
			mm		*mm*
0	0	...	107.2 ± 4.74 (9)	36.4 ± 3.13	69.4 ± 6.65
0	0.125	...	104.7 ± 5.36 (9)	39.1 ± 2.61	69.9 ± 2.30
0	0.25	...	98.4 ± 5.62 (8)	35.1 ± 3.33	77.1 ± 6.86
0	0.375	...	104.9 ± 3.88 (9)	38.7 ± 2.07	71.3 ± 4.67
0	0.5	...	111.0 ± 7.38 (9)	42.1 ± 2.77	58.8 ± 4.94
0	0.75	...	100.8 ± 3.05 (9)	34.7 ± 1.48	71.2 ± 7.59
0	1.0	...	101.9 ± 7.01 (9)	35.9 ± 3.36	68.4 ± 4.88
0	1.5	...	103.8 ± 5.21 (9)	36.0 ± 3.32	65.7 ± 3.12
0	2.0	...	101.9 ± 5.00 (8)	39.8 ± 3.81	72.9 ± 7.09
0	10.0	...	89.8 ± 4.52 (9)	34.9 ± 1.38	73.2 ± 6.49
75	10.0	75 : 1	113.2 ± 4.34 (9)	38.7 ± 2.35	58.3 ± 3.90
100	10.0	10 : 1	108.4 ± 2.17 (9)	33.4 ± 2.41	56.8 ± 4.64
25	0.5		62.4 ± 6.16 (9)	20.8 ± 1.50	31.0 ± 2.85
50	1.0	50 : 1	48.5 ± 6.69 (8)	11.8 ± 1.51	26.8 ± 4.73
75	1.5		55.6 ± 2.46 (9)	12.8 ± 1.18	47.0 ± 7.96
100	2.0		64.9 ± 5.45 (7)	14.1 ± 1.35	48.0 ± 3.85
25	0.25		59.4 ± 4.75 (9)	19.0 ± 1.79	17.2 ± 1.90
50	0.5	100 : 1	51.6 ± 3.65 (9)	8.8 ± 0.64	8.8 ± 0.45
75	0.75		36.7 ± 1.38 (9)	9.1 ± 0.84	10.3 ± 1.15
100	1.0		48.1 ± 3.25 (9)	8.9 ± 0.55	18.8 ± 2.80
25	0.125		39.6 ± 3.81 (9)	11.1 ± 1.18	11.0 ± 1.95
50	0.25	200 : 1	52.6 ± 2.96 (7)	10.7 ± 1.30	10.0 ± 1.84
75	0.375		45.1 ± 4.10 (8)	12.4 ± 0.63	11.0 ± 0.90
100	0.5		36.4 ± 1.98 (9)	6.9 ± 0.80	7.1 ± 0.71
25	0	...	43.1 ± 3.72 (9)	13.8 ± 0.64	15.3 ± 2.47
50	0	...	46.3 ± 9.3 (9)	9.3 ± 0.64	6.0 ± 0.55
75	0	...	36.4 ± 3.10 (9)	7.2 ± 0.64	4.2 ± 0.45
100	0	...	28.9 ± 2.35 (9)	7.9 ± 0.90	5.6 ± 1.05

* $1.0 = 4 \times 10^{-7}$ M.

The data presented above provide good evidence that PABA or PGA, or some related substance synthesized from PABA or PGA is involved in the metabolism of the excised tomato root. If this is the case then it can be expected that, at appropriate inhibitory levels of sulfanilamide, the addition to the medium of substances which require PABA or PGA for their biosynthesis could relieve the inhibition. Therefore, further experiments were carried out to test the effect of such possible substances. Each substance was tested over a wide range of concentrations (0.1 to 200.0 × standard). The concentration of sulfanilamide (100.0 × standard) was such as to give an inhibition of approximately 80 % in growth of main axis.

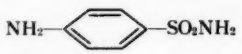
Methionine, in one experiment, relieved the inhibition slightly. Neither adenine, guanine, hypoxanthine, xanthine, cytosine, or uracil affected the inhibition when supplied alone or in the presence of methionine at a concentration which was not itself inhibitory.

DISCUSSION

The assumptions inherent in the experimental method used here for determining the type of antagonism are given elsewhere (25, 26). It is recognized that the validity of the conclusions regarding competitive and noncompetitive inhibition are dependent upon the validity of the assumptions. In spite of the possibility that the assumptions may prove incorrect the results are of definite interest in relation to those obtained by other workers using similar experimental methods with microorganisms. Throughout this discussion the term "folic acid" is used as a collective name for those factors which are active in one or other of the biological tests for "folic acid." The substance n-pteroylglutamic acid, often now termed "folic acid," is referred to as PGA. Its structure and the structure of other substances mentioned in this discussion are shown in figure 3.

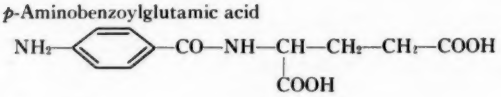
Theories relating to the mechanisms of the inhibitory action of antimetabolites, particularly the sulfonamides, have been discussed and reviewed elsewhere (11, 13, 26). Such inhibitions may be antagonized by substances either structurally similar or dissimilar to the antimetabolite. The theoretical basis upon which the interpretation of such antagonisms are based is contained in the general theory relating to "Inhibition Analysis" (17, 18, 25). The character of an antagonism indicates the metabolic relationship of substances which influence an inhibition. An antagonism may be competitive or noncompetitive. In general a competitive antagonism is interpreted as

Sulfanilamide

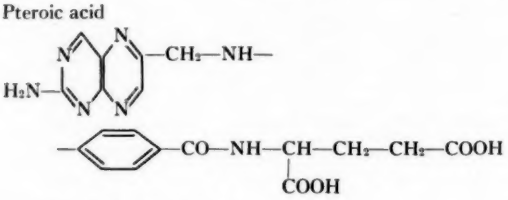


p-Aminobenzoic acid

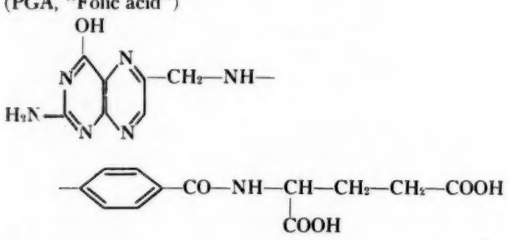
p-Aminobenzoylglutamic acid



Pteroic acid



n-Pteroylglutamic acid
(PGA, "Folic acid")



Folinic acid

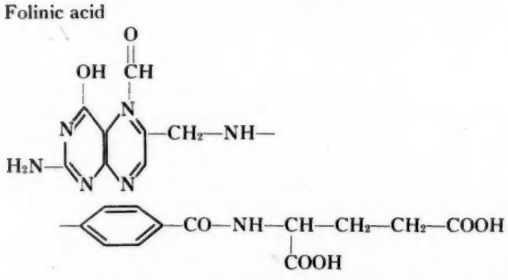


FIG. 3. Molecular structures of substances referred to in the discussion.

showing that the antagonist is a participant, or precursor of such a participant, in the metabolic step inhibited by the antimetabolite. A noncompetitive antagonism is interpreted as showing that the antagonist is a product, immediate or remote, of the metabolic step inhibited by the antimetabolite.

Much of the evidence for the function of PABA and "folic acid" is based upon 1) "Inhibition Analysis," using either sulfonamides or structural analogs of PGA and 2) nutritional studies of microörganisms requiring either PABA or components of "folic acid" for growth. In most cases PABA is a competitive, and PGA a noncompetitive, antagonist of sulfanilamide. Certain other substances, not structurally related to PGA or sulfonamides, and termed secondary antagonists, also relieve the inhibition in a noncompetitive manner. Thus, the most favored general hypothesis is that sulfonamides inhibit the conversion of PABA to PGA and that PGA, or a product thereof, is a coenzyme (Coenzyme F) for processes leading to the biosynthesis of the secondary antagonists. Nutritional studies of the type under 2) above, support this conclusion. There are, however, a number of results which are difficult to fit into this hypothesis.

The results obtained here indicate that both PABA and PGA were competitively antagonistic toward sulfanilamide. Therefore, the results with PABA are in agreement with those generally obtained with microörganisms. It is concluded that PABA is a precursor of, or participant in, the metabolic step blocked by sulfanilamide. The results with PGA are not in agreement with those generally obtained with microorganisms, although instances of competitive antagonism between PGA and a sulfonamide have been reported using *Acetobacter suboxydans* and a Neurospora mutant (12). Pteroic acid was also competitive. In other work (8) *p*-aminobenzoylglutamate was found to be a competitive inhibitor of sulfadiazine with *Streptococcus faecalis*.

On the assumption that "folic acid" is involved in the metabolism of these microörganisms and the tomato root, the following explanations may be postulated to explain these results in terms of the general hypothesis outlined above: 1) Cellular biosynthesis of "folic acid" involves a molecule not identical with PGA, and PGA is not a free compound in the cells. Competitive antagonism of inhibition by sulfonamide could then result if the PGA is first broken down to PABA (or possibly either pteroic acid or *p*-aminobenzoylglutamate) which then enters into competition with the sulfonamide. The breakdown of PGA could occur in the medium, at the cell surface, or in the cell; 2) Cellular biosynthesis of "folic acid" from PABA involves the formation of PGA, and PGA is a free compound, but either PABA has other functions than biosynthesis of PGA, or the PGA supplied in the medium does not reach the site of biosynthesis. Failure to reach the site of biosynthesis could be due to impermeability of the cell to PGA or to destruction of PGA in the medium or the cell. Inhibition by sulfonamide would then be overcome as under 1) above.

Bacillus coli 1883 Co utilizes PABA but not PGA as an anti-sulfonamide. The penetration of the cells of this organism by PABA but not by PGA has recently been demonstrated using tracer techniques (22). With labelled PABA radioactivity was detected in a substance which was active in an assay for "folinic acid" (18). This presumably represents a case where breakdown products, if any, of PGA failed to act as antisulfonamides. However, it has been shown (16) that the activity of PGA as a growth factor for *Lactobacillus arabinosus* was not due to PGA itself but to products of its breakdown in the medium. This supports the above interpretations of a competitive antagonism based upon breakdown of

PGA to PABA or precursor of the inhibited reaction. Support for this view is also given by the fact that the concentration of PGA required to overcome inhibition by a given concentration of sulfanilamide was ten times the concentration of PABA required. However, autoclaving the PGA used here did not increase activity at any level tested. Indeed, at the higher concentrations the activity was decreased by autoclaving. Furthermore, the competitive antagonism between PGA and sulfanilamide was much more clearly shown than that between PABA and sulfanilamide. The lack of complete competition in the case of PABA could be explained if PABA synthesized by the system was having a significant effect on the inhibition. However, at approximately equivalent concentrations of PGA and PABA the incomplete competition observed with PABA was not observed with PGA. This evidence, therefore, indicates that PGA was not being converted into PABA unless PGA was a more efficient source of PABA than PABA itself.

The points discussed above are interpreted as indicating that the activity of PGA with excised tomato roots is not a consequence of its decomposition to PABA. The possibility that PGA is a precursor of the metabolic step blocked by sulfanilamide deserves consideration. Whatever the solution to this problem may be, it is apparent that the "folic acid" metabolism of the tomato root is unlikely to be identical with that suggested for the majority of microörganisms.

The difference between the effect of the same concentration of sulfanilamide, or concentrations of sulfanilamide plus antagonist, on growth of main axis and laterals is of some interest. An explanation is difficult to find but may involve the correlative mechanism whereby the main axis meristem and younger part of the main axis are inhibitory to the growth of lateral meristems (21). Thus, in a medium in which growth of meristems is affected by an inhibitor supplied in the medium, growth of lateral meristems will be a product of 1) the direct inhibitory effect of the inhibitor on the lateral meristem and 2) the relief of the indirect inhibition which is exerted by the main apex on the lateral meristem. This indirect inhibition will be relieved as the inhibitor commences to affect the main axis meristem. In experiments reported here a decrease in growth of lateral roots was observed at concentrations of sulfanilamide, PABA, or PGA which stimulated growth of main axis. The converse was also true at certain concentrations of PABA and PGA. These differential effects on growth of main apex and laterals, and the variability of the system as a whole, may render excised roots, grown with present techniques, unsatisfactory for more critical studies on the mechanism of an antagonism. However, observations of the effect of antimetabolites on the correlation between main apex and lateral growth may yield information on the metabolic basis of the correlative factors.

The failure of various secondary antagonists, including purines, to reverse the inhibition by sulfanilamide was possibly due to the lack of niacin in the medium, or to the high concentration of sulfanilamide. Experiments to test secondary antagonists in the medium containing niacin and with a lower concentration of sulfanilamide will be reported elsewhere.

Summary

Sulfanilamide inhibited the growth of excised tomato roots grown in sterile culture, although stimulation was observed at low concentrations. Both *p*-aminobenzoic acid (PABA) and n-pteroylglutamic acid (PGA) overcame the inhibition. Competitive antagonism was indicated in both cases. In one experiment the inhibition due to excess PGA was relieved by a concentration of sulfanilamide which was itself inhibitory.

In the absence of sulfanilamide both PABA and PGA stimulated growth of main axis and laterals. Stimulation of growth of main axis was not necessarily accompanied by the same effect on laterals, and vice versa. PABA and PGA were inhibitory at lower concentrations than those usually found with microorganisms. Methionine was slightly antagonistic toward sulfanilamide. Various purines and pyrimidines were ineffective either in the presence or absence of methionine.

The results are discussed in relation to the mechanism of inhibition by sulfanilamide and possible relationships of PABA and PGA in the metabolism of the root.

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THE DISTRIBUTION OF RUTIN AND OTHER FLAVONOID SUBSTANCES IN BUCKWHEAT SEEDLINGS[1,2]

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The widespread occurrence of flavonoid compounds suggests that their formation may result from fundamental patterns of plant metabolism. These ubiquitous substances have been the objects of numerous and extensive chemical studies and figured in some of the pioneering work in biochemical genetics. Almost no experimental evidence is available, however, concerning the biogenesis of the flavonoids and the physiological processes in which they may participate.

As has so often been the case, this situation is largely due on the one hand to the unavailability of good chemical methods for small quantities and complex mixtures of the substances, and on the other hand to the paucity of suitable botanical materials. In the first type of difficulty recent developments in analysis, especially in chromatographic and other microchemical techniques, may afford relief. But even if the analytical obstacles should be overcome, the search for an apt plant object to use in attacking the problems of origin and function must involve a preliminary gathering of much descriptive information. For a given candidate the complete flavonoid complement should be characterized; the distribution of each substance within the plant should be known; and the qualitative and quantitative changes in each of the constituents as the plant unfolds its development should be ascertained. The present work with buckwheat is part of an attempt at such a definition of a suitable botanical system.

A number of workers have mentioned the occurrence in the mature buckwheat plant of rutin (3,5,7,-3′,4′-pentahydroxyflavone-3-rutinoside). Schunk (24) discovered this substance in fresh leaves, Wunderlich (28) found it in dried flowers, and Brandl and Schärtel (5) confirmed both reports. Contemporary workers (e.g., 8, 9) have established the occurrence of the glycoside in several species. It has been tentatively suggested (20) that small amounts of other flavonoid substances may also be present in the mature plants. Concerning the flavonoid constitution of buckwheat seedlings no unequivocal information has been available. Nagai (19) reported the occurrence of a small quantity of unknown flavone pigment in etiolated seedlings, while Kuilman (16) and Karstens (15) could not demonstrate flavones in such plants.

This report offers evidence that rutin is present in

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the embryo of the buckwheat seed and that rutin and four other substances of the flavone type occur in both green and etiolated seedlings.

Experimental Methods

Plant Material: Plants of *Fagopyrum esculentum* Moench var. Japanese were employed. Lots of green seedlings were germinated in white sand under greenhouse conditions and harvested after 7 to 10 days growth. Etiolated seedlings were cultured in sand in the dark at 27 ± 2° C; samples of these were analyzed after 3 to 10 days. All cultures received only daily portions of distilled water. One lot of etiolated seedlings, termed "illuminated," was exposed after 5 days to 2 hours of summer sunlight and then replaced in the dark chamber. This lot was taken for analysis 2 days after the period of exposure, by which time anthocyanin pigment existed in the hypocotyls (15, 16). At the time of harvest all plants were divided into their constituent organs. Before germination the hulls were removed from one batch of etiolated seedlings, designed "pericarpless." Ungerminated seeds were soaked in distilled water for 30 minutes and the embryos and endosperms carefully separated for analysis.

Preparation of Extracts: Some fresh plants were extracted with several portions of boiling 95 % ethanol or steeped with this solvent for several days; the crude extracts were concentrated under reduced pressure and used as such. Other fresh plants were ground with boiling water in a Waring blendor for several minutes; these extracts were concentrated under reduced pressure and equal volumes of acetone then added. After removal of the resultant precipitates the acetone solutions were saved for examination. Some of the plant material was dried at 70° C and stored in a desiccator; this was later extracted (Soxhlet) with acetone or 95 % ethanol. In either case the extracts were concentrated at reduced pressure before use. One ethanol extract of fresh etiolated cotyledons and one acetone extract of dried etiolated cotyledons were subjected to hydrolysis with 0.5 % sulfuric acid for 2 hours.

Paper Chromatography of Extracts: Two-dimensional chromatograms of the crude extracts were prepared on sheets of Whatman No. 1 filter paper approximately 23 by 57 cm. After spotting of an extract a sheet was rolled into a cylinder the height of its short dimension and maintained in this posture by stapling with the edges not touching. It was then placed for ascending chromatography in a closed jar. Afterwards the cylinder was unrolled, the edges trimmed, and the paper placed in another chamber for descending development in the long dimension of the sheet. A number of solvent systems were used in various combinations for the two developments, especially isopropanol–water (22–78 or 6–4), acetic acid–water (3–17 or 6–4), or the upper layer of n-butanol-acetic acid–water (4–1–5). The flavonoid spots were located by spraying the chromatograms with 1 % aqueous aluminum chloride and examining them under long-wave ultraviolet light.

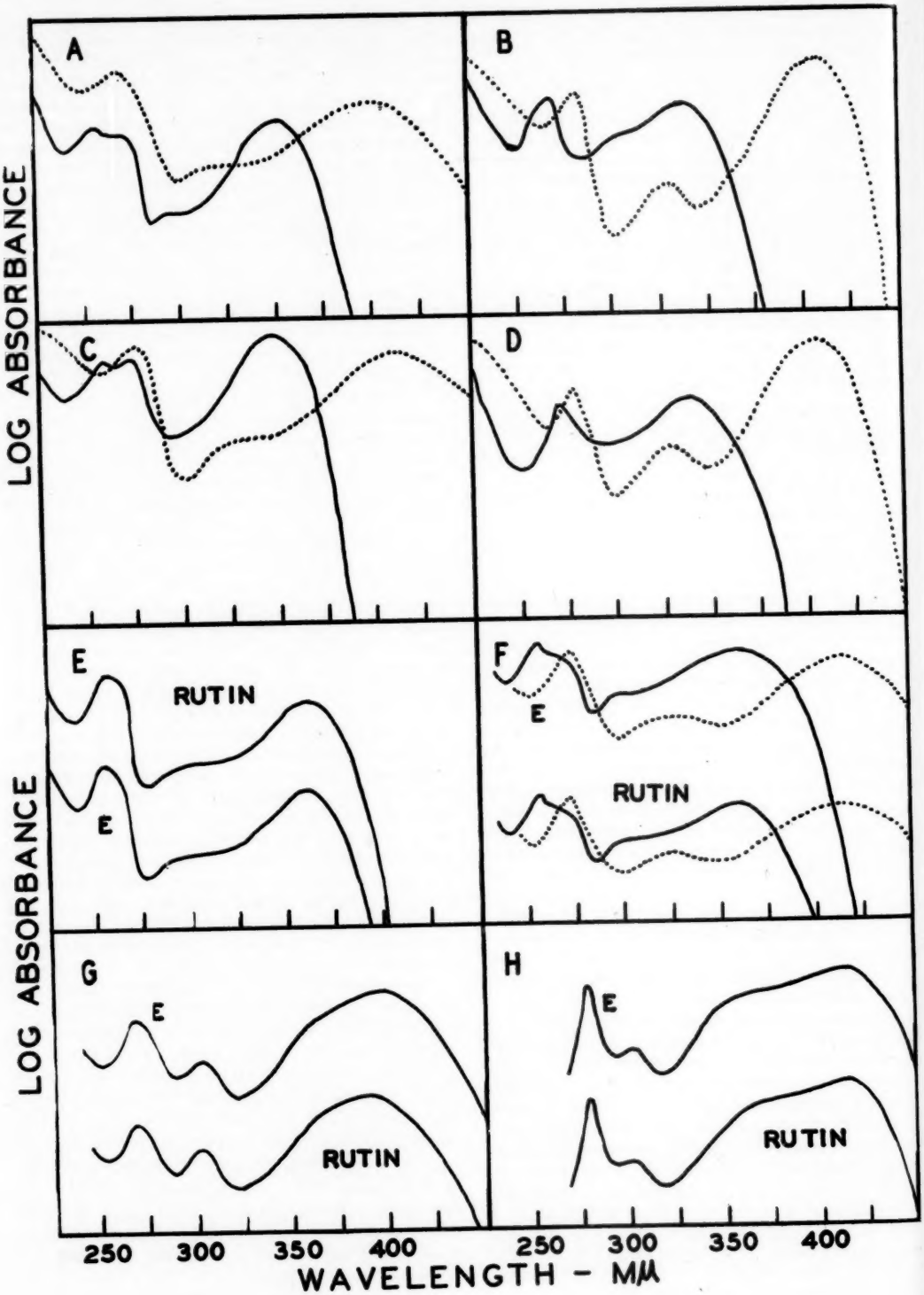
Separation of the Flavonoids: Attempts to separate the buckwheat flavonoids by traditional solvent extraction methods and by ion-exchange (12) and adsorption (14) chromatography were unsuccessful. However, small amounts of the substances—enough for chromatographic and spectrophotometric studies—were separated by repeated applications of a procedure similar to that employed by Nordström and Swain (21). Extracts were spotted in lines on papers which were subjected to extended descending development in one direction with the upper layer of chloroform–isobutanol–water (2–4–4). The pigment zones were located by brief exposure to ammonia vapor and cut apart. Individual substances were removed by leaching with 95 % ethanol for 24 to 48 hours; the combined eluates from a large number of strips were concentrated under reduced pressure to small volumes and used for further studies.

Determination of R_f Values: R_f values of the substances separated by the above procedure were measured in fourteen solvent systems. For each solvent four replicate chromatograms of each substance, of pure rutin, and in some cases of pure quercetin were prepared. Approximately equal quantities of material were spotted, and routine assignments of position on paper, order of spotting, etc., were made by reference to a table of random numbers. The prepared sheets were placed in the chromatographic chamber in contact with the solvent vapors for 24 hours before the descending development in one dimension was begun. In each case the total distance of solvent flow was 40 cm. Pigment zones were located by means of aluminum chloride reagent.

Colors with Reagents: Color reactions of the separated substances were ascertained with a number of spray reagents which have been used with flavonoid compounds. These were 1 % aluminum chloride (11), 2 % zirconium chloride (13), 2 % stannous chloride, 0.5 % magnesium acetate (25), 1 % sodium carbonate (11), Benedict's solution (11), ammonia vapor (3), ammoniacal silver nitrate (3), diazotized *p*-nitraniline plus sodium acetate followed by sodium carbonate (27), 2 % vanillin plus hydrochloric acid (2), and zinc foil plus hydrochloric acid (13). The treated chromatograms were observed under visible and ultraviolet light.

Absorption Spectra of Solutions: Ultraviolet absorption spectra of the separated substances and of pure rutin were measured after first chromatographing them on filter paper. Strips containing the pigments were cut from the chromatograms and the pigments removed by leaching with 95 % ethanol; each eluate was made to 5 ml with additional ethanol. Absorption spectra against solutions from blank chromatograms subjected to the same procedures were read in a Beckman DU quartz spectrophotometer. For spectra in alkaline solution 2.5 ml of an ethanolic solution, the absorption of which had just been determined, were made to 5 ml with 0.1 *N* sodium hydroxide solution (aqueous).

Absorption Spectra of Chromatograms: Ultra-


A
B
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400
WAVELENGTH - MM

violet absorption of substances on filter paper was measured in accordance with the procedure of Bradfield and Flood (4). Strips of paper 0.9 cm wide and 4 cm long containing the spots to be examined were cut from chromatograms; these, together with a similarly treated blank strip, were placed in simple cardboard supports constructed to fit the 1-cm cell holder of the Beckman instrument. At wavelengths smaller than 300 to 310 mμ the selector switch was set at 0.1, the sensitivity knob turned to its extreme counterclockwise position, and the instrument balanced against the blank strip by regulating the slit width with the density knob at zero. At wavelengths greater than 300 to 310 mμ the selector switch could be set at 1.0. Measurements were made in this way on substance E and pure rutin in the untreated state and after spraying the chromatograms with sodium carbonate, aluminum chloride, and zirconium chloride solutions.

Results and Discussion

Characterization of the Substances: Five substances of the flavonoid type were found in buckwheat seedlings. Since the only successful method of separation yielded only very small quantities, the usual chemical studies could not be carried out. Examination of R_f values (table I) of the separated substances confirmed the hypothesis that the five substances are distinctly different. Comparison of these data with published R_f values of flavonoid substances (3, 7, 10, 11, 13, 22, 23, 27) suggested no obvious possible identities for substances A, B, C, and D. It was at once apparent that none of them is quercetin. Hydrolysis of cotyledon extracts resulted in the disappearance of substance E, a result which suggests that it may be the only glycoside present. Chromatograms of the hydrolyzates showed a new spot which resembled quercetin, but this substance was not studied further.

The R_f values of substance E correspond closely with those of rutin, and a statistical test was performed to aid decision in this regard. Differences in mean R_m values (3), where $R_m = \log(1/R_f - 1)$, were calculated for rutin and substance E in the fourteen solvents, and the statistic t was computed to test the hypothesis that the mean difference in R_m in all solvents is zero. The data yielded a value of t equal to −1.586; this was declared not significant at the 5% level (for 13 degrees of freedom $0.20 > P > 0.10$) and the hypothesis of zero mean R_m difference was accepted. It was concluded that the R_m, and consequently the R_f, values of rutin and substance E are not different.

Results of the tests with various spray reagents resembled those reported by others for flavonoid compounds. All five substances were yellow with alkali under visible light, reacted as phenols with diazotized *p*-nitraniline plus sodium carbonate, gave negative vanillin-hydrochloric acid tests, turned red under the zinc-hydrochloric acid reduction, and formed strongly fluorescing yellow or yellow-orange complexes with aluminum, zirconium, and tin. Within this general pattern, the five substances fell into two groups: A, C, and E give similar color reactions, as do B and D. Substance E and authentic rutin displayed identical reactions.

Table I

R_f Values in One-Dimensional Paper Chromatography of Single Buckwheat Flavonoid Substances (A, B, C, D, E) and Authentic Rutin (R). Upper Layers of Two-Phase Systems Were Used, Except Where Indicated. Solvent Constitution Is Designated by Volume

Solvent	A	B	C	D	E	R
EtOAc (H_2O-sat)	0.13	0.31	0.16	0.32	0.13	0.12
EtOAc–AcOH–H_2O (50–2–50)	0.14	0.28	0.19	0.37	0.14	0.14
EtOAc–HCOOH–H_2O (10–2–3)	0.35	0.54	0.51	0.68	0.52	0.53
nBuOH–AcOH–H_2O (4–1–5)	0.47	0.63	0.65	0.78	0.63	0.64
iBuOH–AcOH–H_2O (4–1–5)	0.43	0.63	0.63	0.82	0.62	0.63
Xylene–AcOH (1–3) (H_2O-sat)	0.50	0.62	0.65	0.75	0.72	0.73
Phenol (H_2O-sat)	0.49	0.72	0.64	0.86	0.59	0.57
$CHCl_3$ (H_2O-sat)	0.00	0.00	0.00	0.00	0.00	0.00
$CHCl_3$–iBuOH–H_2O (2–4–4)						
Upper layer	0.15	0.21	0.29	0.43	0.50	0.54
Lower layer	0.09	0.32	0.25	0.68	0.18	0.20
iPrOH–H_2O (22–78)	0.23	0.34	0.45	0.59	0.64	0.63
iPrOH–H_2O (6–4)	0.55	0.69	0.76	0.85	0.82	0.83
AcOH–H_2O (3–17)	0.22	0.33	0.39	0.54	0.56	0.57
AcOH–H_2O (6–4)	0.50	0.62	0.64	0.74	0.71	0.72

The ultraviolet absorption spectra of the five substances in chromatogram eluates strengthen the conclusion that all are flavones or flavonols. These are depicted in figure 1 (A–E) with the logarithm of absorbance plotted against wavelength in order to remove the effect of concentration, which is unknown in all cases. Since measurements were made on the same eluates in both ethanolic and alkaline solutions, it is possible to compare in a relative way the heights of the absorption peaks under these conditions (18). A summary of spectral relations is presented in table II.

The shifts of the absorption maxima to longer wavelengths under alkaline conditions indicate that all five substances possess free phenolic groups (17). In both ethanolic and alkaline solutions substances B and D display spectra very similar to those reported for some derivatives of apigenin (18). Substances A

Fig. 1. Ultraviolet absorption spectra of buckwheat flavonoid substances. A–E. Spectra of chromatogram eluates: solid lines, in 95% ethanol; broken lines, in alkaline solution, corrected for volume changes. A–D. Substances A, B, C, and D, respectively. E. Substance E and authentic rutin. F–H. Spectra of zones of authentic rutin and substance E on filter paper chromatograms. F. Solid lines, untreated; broken lines, with 1% sodium carbonate. G. With 2% zirconium chloride. H. With 1% aluminum chloride.

and C, on the other hand, show in ethanol the low peak or shelf in the region 290 to 310 mμ characteristic of flavonols (1, 26), and the spectra of these two strongly resemble those reported by Briggs and Locker (6) for some substituted derivatives of quercetin. The stability of A, B, C, and D in alkaline solution suggests that none of these substances possess an ortho-diphenolic configuration.

In ethanol, rutin and substance E display identical spectra. Because rutin is notoriously unstable in alkaline solution, the data given in table II for this compound are not of exact significance; the height and possibly the location of the Band I maximum were observed to change with time. It is important to note, however, that this behavior was also displayed by substance E: at the times of measurement the locations of the maxima in alkali were the same as those of rutin, and the relative heights of the peaks quite similar. The aluminum complexes of rutin and substance E also showed identical spectra in solution, with maxima at 400 and 271 mμ.

Results of the spectral measurements for rutin and substance E on filter paper are shown in figure 1 (F–H), with the logarithm of absorbance again plotted against wavelength to eliminate unknown concentration effects. The two substances show identical spectra in the untreated state as well as with the three spray reagents used. Both showed maxima in the following locations: untreated, 362 and 257 mμ; with 1% sodium carbonate, 420 and 273 mμ; with 2% zirconium chloride, 407 and 272 mμ; and with 1% aluminum chloride, 405 and 277 mμ.

From the close correspondence of R_f values in a number of solvents, the identical color reactions, and the similarity in ultraviolet absorption spectra under a variety of conditions, it seems apparent that substance E is rutin. Similarly, on the basis of the color tests and absorption measurements it is concluded that substances A, B, C, and D are flavones or flavonols.

TABLE II

LOCATIONS AND RELATIVE HEIGHTS OF ABSORPTION MAXIMA OF BUCKWHEAT FLAVONOID SUBSTANCES (A, B, C, D, E) AND AUTHENTIC RUTIN (R) IN 95% ETHANOL AND IN ALKALINE SOLUTION. A. ABSORBANCE; A_0. ABSORBANCE AT λ MAX OF BAND I IN 95% ETHANOL (λ MAX IN mμ). VALUES FOR ALKALINE SOLUTIONS CORRECTED FOR VOLUME CHANGES

	A	B	C	D	E	R
Band I						
Ethanol						
λ max	351	333	353	337	362	362
$A_0 = 100$	100	100	100	100	100	100
Alkali						
λ max	409	399	415	402	408	408
A as % of A_0	109	136	86	148	70	79
Band II						
Ethanol						
λ max	257	270	258	272	257	257
A as % of A_0	97	107	80	95	120	112
Alkali						
λ max	269	279	270	279	269	269
A as % of A_0	147	110	93	107	120	109

TABLE III

OCCURRENCE OF FLAVONOID SUBSTANCES IN BUCKWHEAT SEEDS AND SEEDLINGS. A, B, C, D. UNKNOWN FLAVONOIDS. R. RUTIN. P. PRESENT. N. NOT PRESENT

	A	B	C	D	R
Ungerminated Seed					
Pericarp	P	P	P	P	P
Endosperm	N	N	N	N	N
Embryo	N	N	N	N	P
Green Seedlings					
Root	N	N	N	N	P
Hypocotyl	N	N	P	N	P
Cotyledon	P	P	P	P	P
Pericarp	P	P	P	P	P
Etiolated Seedlings					
Root	N	N	N	N	P
Hypocotyl	N	N	N	N	P
Cotyledon					
Untreated	P	P	P	P	P
Hydrolyzed	P	P	P	P	N
Pericarp	P	P	P	P	P
"Pericarpless" Seedlings					
Root	N	N	N	N	P
Hypocotyl	N	N	N	N	P
Cotyledon	P	P	P	P	P
"Illuminated" Seedlings					
Root	N	N	N	N	P
Hypocotyl	N	N	N	N	P
Cotyledon	P	P	P	P	P
Pericarp	P	P	P	P	P

OCCURRENCE OF THE SUBSTANCES IN BUCKWHEAT SEEDLINGS: Results of the preliminary paper-chromatographic survey of the distribution of the five flavonoid substances in buckwheat seedlings are presented in table III. The same qualitative picture was obtained with both fresh and dried material, regardless of the method of extraction used. In the ungerminated seed only rutin was present, and this was located entirely in the embryo. A fairly rapid elaboration of substances A, B, C, and D, and probably rutin must therefore have occurred during germination since some of the samples were harvested after only 3 days growth.

Green and etiolated seedlings showed the same pattern with only one exception: substance C, not found in etiolated hypocotyls, was present in those of green plants. The "illuminated" seedlings, hypocotyls of which were red with anthocyanin pigment, showed no qualitative difference from other etiolated seedlings.

The cotyledons always contained all five substances, while A, B, C, and D were never found in the other organs with the one exception noted. Rutin, on the other hand, seemed to be present in all parts of the plant, although the amounts in the roots were quite small. The pericarps always contained all five; the fact that this structure properly belongs to the preceding generation suggests that all of the substances may also occur in parts of mature plants. The presence of all five in seedlings from which the pericarps were removed before germination precludes

the possibility, unlikely on other grounds, that the appearance of the substances in the plants proper was the result of translocation from the pericarps.

Summary

Five flavonoid substances have been found to occur in both green and etiolated buckwheat seedlings; their distribution in roots, hypocotyls, cotyledons, and pericarps is described. Only small quantities of the substances have been separated, but these have been sufficient for determination of R_f values in a number of solvents, performance of color tests, and measurement of ultraviolet absorption under various conditions. One of the five substances was shown by comparison with an authentic sample to be rutin; it is the only one found in the ungerminated seed, as well as the only one present in all parts of developing seedlings. Properties of the other four substances suggest that all are flavones or flavonols. Free quercetin was not detected in seeds or seedlings.

Grateful appreciation is expressed to Professor R. F. Dawson, under whose direction the described research was completed. Dr. Joseph Naghski of the United States Department of Agriculture very kindly supplied authentic samples of rutin and quercetin.

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ASCORBIC ACID OXIDASE IN BARLEY ROOTS[1,2]

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Two possible terminal metallo-oxidases have been found in barley, ascorbic oxidase by James and Cragg (14) and cytochrome oxidase by Merry and Goddard (23). Studies on the intracellular distribution of various ascorbic oxidases by Mandels (21) and Newcomb (25) indicated that the enzymes are localized at the surface. Newcomb (25) has shown that in many higher plants ascorbic oxidase is attached to or situated near the cell wall. According to the reviews of Goddard and Meeuse (7) and Goddard and Stafford (8), cytochrome oxidase is localized in the mitochondria. Thus, it may be possible to distinguish between ascorbic oxidase and cytochrome oxidase by their intracellular distribution. However, there are differing views on the intracellular distribution of cytochrome oxidase in higher plants. Lundegårdh (18) postulated a coating of cytochrome oxidase on the inner surface of the cell walls and in the tonoplast membrane of wheat roots, while Butler (3) considered that it is localized both in the tonoplast membrane and in the mitochondria. Lundegårdh (18) suggested that the peripheral localization facilitates salt respiration and accumulation which have been found associated with the cytochrome system (17, 31). An added complication raised by James (12) questions the existence of a functional cytochrome oxidase in root tips from older barley seedlings. Consequently, it was of interest to determine the identity of a barley root enzyme utilizing ascorbic acid and its localization in the cell.

METHODS AND MATERIALS

Barley grains, varieties Moore and Atlas 46, were germinated following the methods of Machlis (20). The entire procedure was carried out at 28° C and 90 % relative humidity in a dark room occasionally illuminated by red light. Roots from the barley seedlings were harvested 5 days after soaking the grains. Segments 5 mm long were cut from the roots, washed with distilled water, and stored for ca 1 hour at 7° C in distilled water.

Homogenates of the root segments were prepared essentially by the method of Newcomb (25). Cell-free homogenates (25 % w/w) were obtained after grinding the roots for 3 to 5 minutes in either ice-chilled water or media initially 0.8 M tonicity and adjusted to 0.4 M final exogenous tonicity.

Centrifugal fractionations of the homogenates were carried out in a refrigerated centrifuge at 0 to 2° C. The wall fragments were resuspended three times in the appropriate medium and the fragments resedimented, for 5 minutes at $225 \times g$ if not otherwise noted. The washed preparations were then resuspended to their original volumes. Recovery of activity was determined by assay of equivalent portions of the fractionated homogenate.

Ascorbic oxidase and cytochrome oxidase were assayed by Warburg manometric procedures. Temperature equilibration was carried out for 30 minutes before the assay. Substrates and other reagents were then tipped into the main vessel compartments which contained 0.5 ml enzyme preparation, unless otherwise specified. Concentrations of reagents before and after substrate addition were maintained constant. Exogenous inorganic ions were usually excluded. For example, ascorbate was added as the acid form and not as the sodium or potassium salt. This accounts for the low pH in experiments completed before tris-hydroxymethyl-amino-methane (Tris.), an organic base, was available. In later experiments sufficient organic base was added to maintain the pH. The final pH values of the assay solutions after the last manometer readings were determined with glass-calomel electrodes.

The activities quoted are corrected for non-enzymatic oxidation of substrates as computed from similar treatments either without the enzyme preparations or with heat inactivated preparations. No effects of tonicity on the activity were found. Consequently, precautions were not taken to assay activity under a given tonicity.

Preparations of various ages were employed since the homogenate was relatively stable with more than 70 % of the activity remaining after 10 days at 7° C.

The gases for experiments on carbon monoxide effects were commercial oxygen and nitrogen, and carbon monoxide generated from formic acid and washed through 6 M KOH. Gas mixtures were made up in 20-liter bottles with a gas displacing solution saturated with Na_2SO_4 and made acid to methyl red with sulphuric acid. The manometer vessels were gassed with 1 liter of the appropriate mixture while being shaked in the temperature bath.

The light source for the attempted reversal of carbon monoxide inhibition was a tungsten filament cylindrical bulb of intensity 1×10^2 fc at the manometer vessel position.

The nitrogen estimations were carried out according to the modified Nessler procedure of Umbreit et al (29).

EXPERIMENTAL RESULTS AND DISCUSSION

ASCORBIC OXIDASE IN ROOT HOMOGENATES: Stimulation by cytochrome c of an enzymatic oxidation of ascorbic acid may be considered as evidence for the presence of cytochrome oxidase. (In the assay for cytochrome oxidase, ascorbic acid is preferred by Slater (27) to hydroquinone or *p*-phenylenediamine

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because the latter produce toxic effects and non-enzymatic oxidations after their initial dehydrogenations. It has been reported by Friedkin and Lehninger (6) that oxidative phosphorylations do not occur with hydroquinone or *p*-phenylenediamine whereas a low rate of phosphorylation accompanies the oxidation of ascorbic acid by cytochrome.) An oxygen uptake by some root homogenates stimulated by cytochrome c suggested the presence of cytochrome oxidase (fig 1). The total oxygen uptake in this experiment was less than the oxidative equivalent of the added ascorbate, being 87 % and 91 % of the expected, with and without cytochrome c, respectively. In other experiments oxygen uptake equivalent to a complete oxidative reaction was obtained. Many other preparations showed only a high native activity with ascorbic acid and no extra activity with cytochrome c. Therefore, it was questionable whether the activity could be ascribed solely or even partially to cytochrome oxidase. A lack of stimulation owing to a sufficiency of a bound-cytochrome c system was unlikely. No stimulations were observed with these preparations which were homogenized in water, a treatment almost certain to elute cytochrome c from the cytochrome system. Preliminary experiments thus showed in all cases a marked native "ascorbic oxidase" and in some cases an additional "cytochrome oxidase" activity.

TABLE I

UTILIZATION OF SUBSTRATES BY ASCORBIC OXIDASE IN THE GROSS HOMOGENATE

TREATMENT *	μL O_2 UPTAKE/60 MIN
Ascorbate **	86
Ascorbate + cyt c	125
p-Phenylenediamine **	1
p-Phenylenediamine + cyt c	2
Hydroquinone **	2
Hydroquinone + cyt c	+7

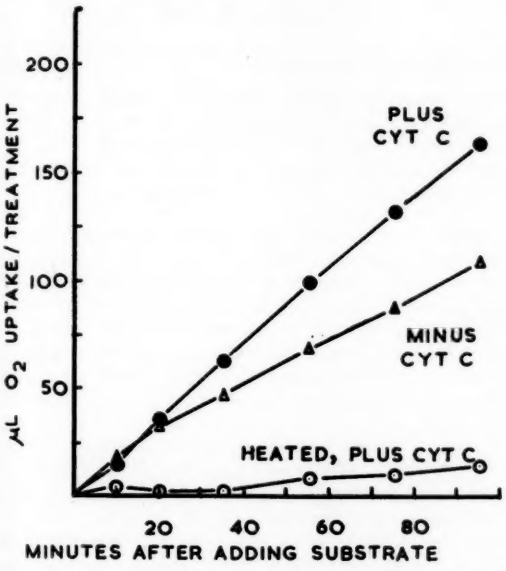
Moore roots were homogenized in 10 % gum ghatti + 0.1 M mannitol (38 % w/w, fresh wt). Activities of the fresh preparation (Expt. R-150 B) were assayed at final pH 6.7 to 6.9 and 25° C.

* Additional reagents were K-maleate, 4.6×10^{-2} M, adjusted to pH 7, and cyt c, 1.0×10^{-4} M.

** Concentration in 3.5 ml final volume was 6.4×10^{-3} M.

Characterization of the enzymatic activity was carried further by testing the substrate specificity, inhibition by copper enzyme and iron enzyme poisons, and the pH tolerance of the enzyme during homogenization.

Many preparations utilized only ascorbic acid as substrate (table I). These data suggest the absence of active cytochrome oxidase and constitute positive evidence for ascorbic oxidase. The stimulation by



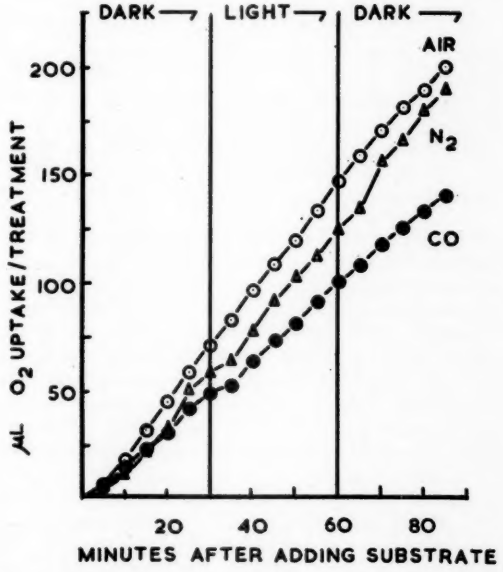


FIG. 1 (*left*). Apparent "cytochrome oxidase" in the gross homogenate. Moore roots were homogenized in water and yielded a 22.7 mg dry wt/ml gross homogenate. Activities of the 4-day-old homogenate (Expt. R-51) were tested at final pH 5.0 and 25° C. Substrate in 2.5 ml final volume was ascorbic acid, 7.5×10^{-3} M. Additional reagents were glucose, 2 %; cytochrome c, 1.7×10^{-5} M; and nicotinamide (NA), 1.0×10^{-2} M.

FIG. 2 (*right*). Inhibition of ascorbic oxidase in the gross homogenate by carbon monoxide. Moore roots were homogenized in water and yielded a 22.5 μM N/ml gross homogenate. Activities of the 8-day-old homogenate (Expt. R-174) were tested at final pH 5.8 to 5.9 and 20° C. Substrate in 2.0 ml final volume was ascorbic acid, 1.0×10^{-2} M. Additional reagents were maleic acid, 2.2×10^{-2} M; and TRIS, 2.4×10^{-2} M. Gas mixtures were air, N_2 90 % + O_2 10 %, and CO 90 % + O_2 10 %.

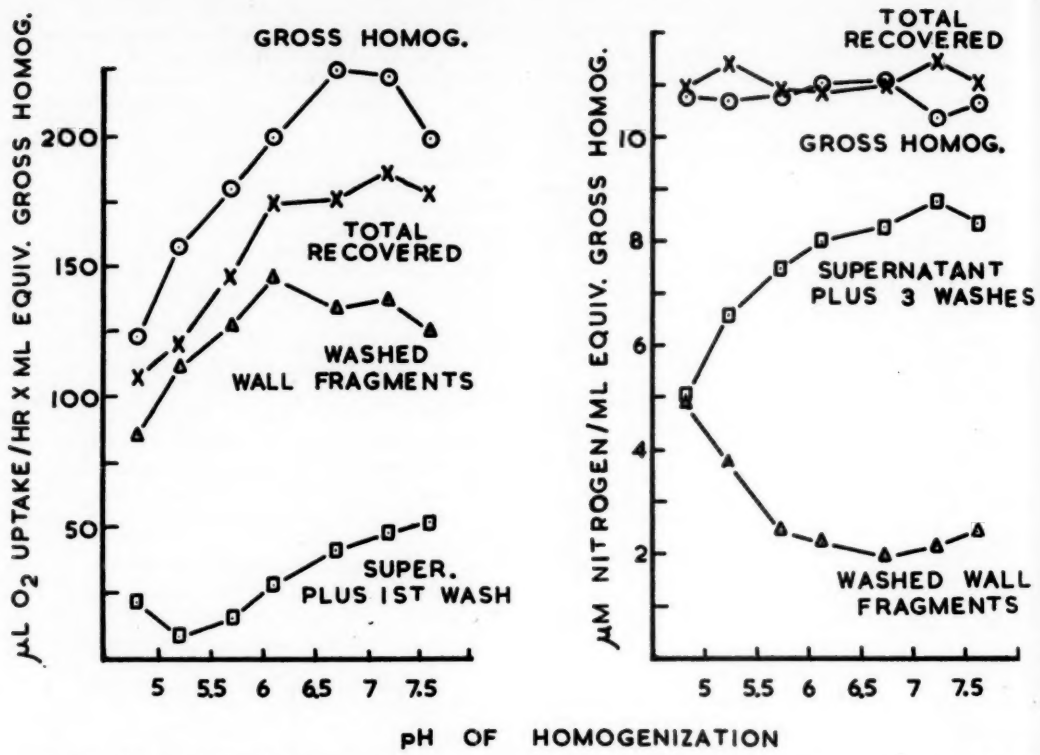


FIG. 3. Effect of pH of homogenization on the partitioning of ascorbic oxidase and nitrogen content. Atlas 46 roots, 390 segments of 10 mm per treatment, were homogenized in 0.2 M phosphate buffer and diluted to 0.1 M phosphate buffer. Fractionation was carried out in the usual manner. Activities of the fresh preparations (Expt. H-245) were tested at final pH 5.5 to 5.9 and 25° C. Substrate in 1.5 ml final volume was ascorbic acid, 1.1×10^{-3} M. Additional reagents were maleic acid, 8×10^{-2} M; and TRIS, 1.2×10^{-1} M.

cytochrome c (table I) was apparently not specific since ferric tartrate also accomplished equal, if not greater results. The action of tartrate, and perhaps of cytochrome c, presumably was to remove inhibitory substances by complex formation (cf. the effects obtained with complexing agents by Altman and Crook (1)). Perhaps the cytochrome c stimulations were non-specific protein effects. Thus, the difference shown in figure 1 may have resulted from a stabilization of ascorbic oxidase rather than a stimulation of cytochrome oxidase.

Activity of the gross homogenates was decreased by carbon monoxide to 80 % of the nitrogen control. This inhibition was not reversed by light (fig 2). The lack of reversibility by light is evidence against this activity being mediated by cytochrome oxidase. The ascorbic oxidase studied by Matsukawa (22) similarly possessed a sensitivity to carbon monoxide which was not light reversed.

Copper complexing agents such as azide, sodium diethyldithiocarbamate (DDC), cyanide, and 8-hydroxyquinoline (8-OH-Q) inhibited the activity (see fig 6 for the type of results obtained). The amount of DDC present in the reaction mixture was not enough to compensate for oxygen uptake by its decomposition into carbon-oxy-sulfide under acidic conditions (15). Lack of inhibition by *p*-nitrophenol at 1.33×10^{-4} M apparently rules out tyrosinase as a possible enzyme present sensitive to 8-OH-Q or DDC.

The pH tolerance of the enzyme was determined while studying the effects of pH on the fractionation of homogenates. Above and below the pH range 6.7 to 7.2 the enzyme was inactivated to various degrees as shown by assay in the optimal pH range (see activities of the gross homogenates, fig 3). The optimal pH region for homogenization is distinct from that for activity which occurs between pH 5 and 6. Inactivation by low pH is one property of ascorbic oxidase (5). Consequently, the type of cytochrome c stimulation, or maintenance of activity, shown in figure 1 may have resulted from a protective action against the acidic conditions of the assay.

The above results, although individually inconclusive, all point to ascorbic oxidase as the source of activity towards ascorbic acid. The most convincing single point for this is the enzyme specificity for ascorbate.

CYTOCHROME OXIDASE IN ROOT HOMOGENATES:

The failure to detect cytochrome oxidase in the majority of preparations may have resulted from several factors.

Cytochrome oxidase may be present in such low amounts in roots that under conditions of the assay 0.5 ml of the gross homogenates, representing at most 380 mg of roots, was not sufficient to exhibit activity. A masking of cytochrome oxidase by a greater activity of ascorbic oxidase is possible.

The enzymes may have been inactivated by osmotic fluctuation, inhibitor action, or by acidic conditions. Osmotic fluctuations may have had little effect, although the mitochondrial system is noted for its tonicity requirements for activity (16, 19). No precautions against osmotic variations were taken in the majority of experiments since chemical reduction of cytochrome c obviates the need for better structural integrity of the mitochondrion in the cytochrome oxidase assay. On the other hand, both inhibitor action and especially the acidic conditions probably contributed to cytochrome oxidase inactivation. An inhibitor of cytochrome oxidase has been detected in preparations of particles from barley roots (10). The acid pH of homogenization and assay may have inactivated cytochrome oxidase to a greater extent than ascorbic acid. In this event the amount of tissue used would have been critical for demonstration of cytochrome oxidase. It should be reiterated that the system was studied as far as possible without exogenous salts. Consequently, in many preparations the absence of buffer determined the acidic conditions of homogenization and assay unfavourable for cytochrome oxidase.

A possible transient cytochrome oxidase in barley may have been a reason for erratic detection of the enzyme. James (12) did not find the cytochrome system in root tips of 10-day-old barley seedlings either by spectrophotometric means or enzyme inhibitor studies. On the other hand, cytochrome oxidate was detected in root tips from 3-day-old barley by James (13). The development of roots from the 5-day-old barley used in the experiments may have been on the borderline for the amount of enzyme detectable by the assay. The possible transcience is likely to be different from that of succinoxidase isolated by Goodwin and Waygood (9) from barley seedlings since cytochrome oxidase was always detected in their preparations. It is possible also that the bulk of their cytochrome oxidase is from the shoot and not from the barley root.

A situation similar to that for barley exists for older wheat roots from which Waygood (30) and Butler (4) were not able to extract an active preparation of cytochrome oxidase. The difficulty appears to be one of method since Lundegårdh (18) has shown spectrophotometrically the existence of a functioning cytochrome system in intact wheat roots.

The technique for extraction and assay of Millerd et al (24) applied to 5-day-old roots yielded a "mitochondrial" preparation which on an absolute oxygen uptake basis was weakly active. However, it possessed a $Q_{O_2}(N)$ of 130 with succinate and 280 with *p*-phenylenediamine plus cytochrome c. It is clear that under certain conditions of extraction and assay, cytochrome oxidase may be demonstrated in roots from 5 day old seedlings. A method for extraction and assay for cytochrome oxidase and succinoxidase from roots of older barley of another variety is reported separately (10).

Localization of Ascorbic Oxidase: As Newcomb (25) found for many higher plants, ascorbic oxidase of barley root cells was peripherally localized. Three lines of evidence were obtained from barley roots in corroboration of Newcomb's findings: (1) the partitioning of nitrogen content and ascorbic oxidase by homogenization and centrifugal fractionation; (2) the change in recovery of activity with the wall fraction induced by plasmolysis of the root cells; and (3) the effect of external solution pH upon ascorbate-induced oxygen uptake of root segments.

If homogenization does not change the distribution of enzymes in situ, then centrifugal fractionation should reveal the localization of ascorbic oxidase. The

Table II

Recovery of Ascorbic Oxidase in the Cell Wall Fraction

Expt.	Fraction	Activity recovery %	μM N/ml	$Q_{O_2}(N)$
R-160 Moore	Gross *	...	31.7	1070
"	Wall fragments, unwashed	90	16.5	1890
R-171 Moore	Gross *	...	18.7	965
"	Wall fragments, 3× washed	98	8.6	2070
R-167 Moore	Gross **	...	22.5	1320
"	Wall fragments, 3× washed	100	6.6	4700
HR-216 Moore	Gross **	...	30.2	1280
"	Wall fragments, 3× washed	91	3.6	9800
HR-220 Moore	Gross **	...	11.9	2040
"	Wall fragments, 5× washed	70	3.0	5670
HR-239 Atlas 46	Gross **	...	10.3	751
"	Wall fragments, 3× washed	74	0.9	6360
HR-242 Atlas 46	Gross **	...	16.0	772
"	Wall fragments, 7× washed	74	5.4	1690

* Homogenized in mannitol.
** Homogenized in water.

ascorbic oxidase activity of the gross homogenate was found to be easily sedimented under low centrifugal forces. The recovery of activity with the wall fragments ranged from 79 to 94 % under 9 to 560 × g, respectively. The high and relatively constant recovery of activity, although the nitrogen content of the wall fraction itself was low and variable (table II), is evidence for the peripheral localization. The specific activities varied presumably because different amounts of nitrogen other than that of ascorbic oxidase were removed from the wall. However, under the conditions of the homogenization ascorbic oxidase itself may be adsorbed from the cytoplasm onto the wall fragments through an isoelectric or precipitation effect. Wildman and Jagendorf (32) have pointed out the precipitating effect of low pH which may occur during the mixing of the vacuolar contents with the cytoplasm.

To determine a possible isoelectric effect, barley roots were homogenized in phosphate buffers of various pH values and the distribution of activities and nitrogen in the various fractions were determined. Enzyme assays were made in the optimal range of pH which was previously determined. To compare strictly the pH effect only root segments of similar development were used, i.e., segments from the same regions measured from the root apex. An isoelectric effect is characterized by a minimum protein solubility near the isoelectric point and increased solubility on both sides of it. The absence of a peak of recovery of nitrogen with the wall fragments probably is evidence against an isoelectric effect (fig 3). The data do show an acid-induced precipitation of nitrogen containing substances on the wall fragments but the trend is opposite to that for recovery of activity. However, the nitrogen content is a relatively gross means of detecting distribution changes affecting a single enzyme. Thus, the high recovery peak of activity and the low peak in the non-wall fractions may indicate a displacement of ascorbic oxidase. Nevertheless, if an isoelectric effect were present it was apparently negligible since these peaks do not coincide and especially as a relatively constant percentage of recovery was found in the wall fractions. There was a small but sharp increase in recovery between pH 6.7 and 6.1 from ca 60 to 70 % of the gross homogenate activity. Above and below this pH range, enzymatic recovery was constant at the percentage noted. The impracticability of determining the activity of the second and third wash solutions contributed to an incomplete recovery in the various fractions.

Waygood (30) found that dilute salt solutions eluted ascorbic oxidase from the residue of the centrifuged homogenate of wheat seedlings. The residue undoubtedly contained cell wall fragments. The effect noted by Waygood possibly was superimposed upon the effect of pH. The enzyme recovery in the barley root wall fractions was ca 90 % for homogenates prepared in water compared to the 70 to 60 % recovery for homogenates prepared in phosphate buffers at the corresponding pH (between pH 6 and 6.5).

TABLE III

EFFECT OF PLASMOLYSIS IN LOWERING THE RECOVERY OF ASCORBIC OXIDASE WITH THE CELL WALL FRAGMENTS OF ROOTS

FRACTION	MEASUREMENTS	SOAK TREATMENT	
		WATER	1.5 M GLUCOSE
Gross homogenate	μM N/ml	11.2	11.4
	$Q_{O_2}(N)$	1100	979
Wall fragments, 3 × washed	μM N/ml	4.2	4.0
	$Q_{O_2}(N)$	2650	2000
	Activity recovery	90 %	72 %

Moore roots were presoaked in 1.5 M glucose or in water for 1 hr, rinsed, and then homogenized in water. The wall fragments were sedimented under 125 × g for 5 min. Activities of the fresh preparation (Expt. HR-231) were assayed at final pH 5.7 and 25° C. Substrate in 1.5 ml final volume was ascorbic acid 1.0×10^{-2} M. Additional reagents were maleic acid, 4×10^{-2} M, and TRIS, 6×10^{-2} M.

The high recovery with the cell wall fragments, in spite of the salt elution, and the absence of a significant isoelectric effect in the fractionation of homogenates suggest the peripheral localization of ascorbic oxidase.

If ascorbic oxidase were peripherally localized any treatment which separates the cytoplasm from the cell wall prior to homogenization should tend to decrease the recovery of activity with the wall fractions. However, if current concepts of the intermingling of peripheral cytoplasm with the cell wall are correct (26) plasmolysis should not greatly affect the distribution of ascorbic oxidase. The data in table III show that a hypertonic soak treatment of the root segments resulted in a decreased yield with the cell wall fragments. The small decrease in recovery is in accord with an intimate association of ascorbic oxidase with the cell wall. A coincidence to note is that the decrease in recovery is of the same order of magnitude as the salt elution effect, about 20 %.

The surface localization of an enzyme subjects it to a greater influence of the external environment. As Mandels (21) proposed, the pH of external solution would thus markedly affect a surface localized ascorbic oxidase. The evidence of Hope (11) and Butler (3) have affirmed the earlier conclusion of Brooks and Moldenhauer-Brooks (2) as to the freely permeable character of the cell cytoplasm to ions. However, the cytoplasmic proteins probably are capable of buffering externally added hydrogen ions. Consequently, an ascorbic oxidase activity of intact roots as a function of the external solution pH may be regarded as evidence for the surface localization of ascorbic oxidase. In the comparison of roots for the

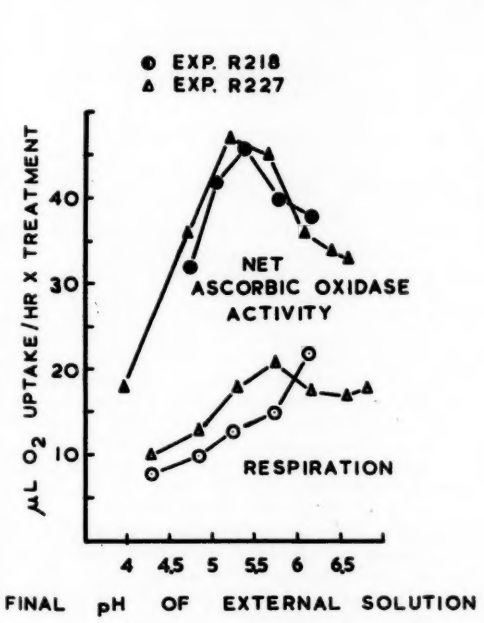
pH effect, only segments from similar regions were tested. As shown in figure 4 oxygen uptake by roots supplied with ascorbate was greatly affected by external solution pH. Moreover, the optimal pH range was within 5.0 to 6.6 which has been reported as optimal for various ascorbic oxidase preparations (5). The pH is not considered to act through its effect on the permeability of ascorbic acid. The mono-nodal activity-pH curve cannot be predicted from a consideration of pH, the pK of ascorbic acid, and the effect of pH on the weak electrolytes of the cytoplasm. Thus, the activity-pH relationship agrees with a peripheral localization of ascorbic oxidase. The assumption is made that the activity is due substantially to ascorbic oxidase and is not, for example, a consequence of ascorbate reaching cytochrome oxidase.

The peripheral localization of ascorbic oxidase found by Newcomb (25) for many higher plants is supported by the lines of evidence given above. A surface localized enzyme is not without precedent. The failure to localize ascorbic oxidase prior to the efforts of Newcomb can probably be attributed to the lack of interest in the enzymatic properties of the wall fragments and to the fortuitous release upon homogenization of ascorbic oxidase sufficient to provide an enzyme source.

It appears that substantially all the ascorbic oxidase is associated with the cell wall in barley roots which were soaked in water prior to homogenization. Salt solutions can elute ascorbic oxidase from the cell periphery. It was not established that the elution occurred after homogenization. It may be possible that elution in vivo occurs.

Identification of the Wall-bound Ascorbic Oxidase: It has been tacitly assumed in the previous section that the activity isolated with the wall fragments is attributable to only the ascorbic oxidase in the gross homogenate. However, active cytochrome oxidase on the wall would give similar results. The properties of the wall-bound enzyme were examined and found to agree with those of ascorbic oxidase. The pH optimum for activity conformed to the previously reported optimum for ascorbic oxidase (5) and the activity showed no cytochrome c stimulation even with preparations homogenized in water (fig 5). The system was inhibited by copper complexing agents (fig 6).

One property of the wall-bound enzyme differs



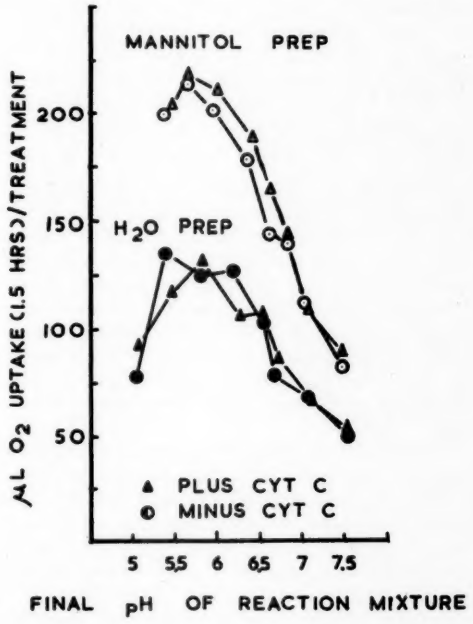


Fig. 4 (*left*). Effect of external solution pH on oxygen uptake by root segments supplied with ascorbic acid. Moore roots were stored 2 to 3 days in distilled water at 7° C. Activities of 175 segments of 5 mm (Expt. R-218) and 60 segments of 10 mm (Expt. R-227) per treatment were tested in 2.5 ml final volume at 25° C. The assay medium consisted of ascorbic acid, 6.9×10^{-3} M; TRIS, 6.6×10^{-3} M; and various amounts of a TRIS-maleate buffer ranging from 4.0 to 7.5×10^{-2} M TRIS and maleic acid 1.9 to 4.0×10^{-2} M.

Fig. 5 (*right*). pH activity curve of the wall-bound ascorbic oxidase. Moore roots were homogenized in water and 0.8 M mannitol. Nitrogen content of the water preparation was 2.2 μM N/ml wall fraction. Activities of 4- and 5-day-old preparations (Expt. R-176) were tested at 25° C. Substrate in 1.5 ml final volume was ascorbic acid, 2.3×10^{-2} M. Additional reagents were cyt c, 1.0×10^{-5} M; and TRIS-maleate buffer ranging from 1.23 to 1.99×10^{-1} M TRIS and 3.6 to 10.0×10^{-2} M maleic acid.

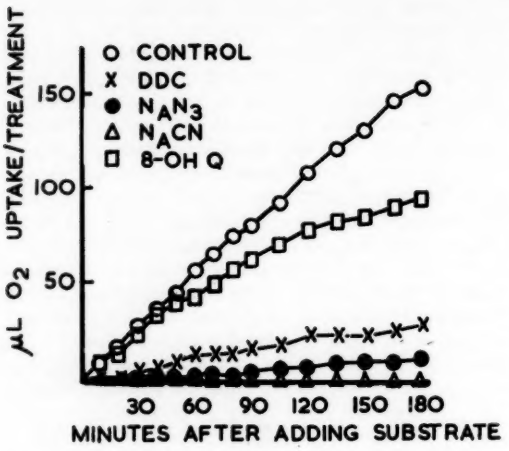


FIG. 6. Inhibition by copper complexing agents of the wall-bound ascorbic oxidase. Moore roots previously soaked in 1.5 M glucose for 1 hr were homogenized in water. The wall fraction was sedimented under $125 \times g$ for 5 min and contained 4.0 μM N/ml wall fraction. Activities of the 1-day-old preparation (Expt. HR-232) were tested at final pH 5.4 to 5.7 and 25° C. Substrate in 1.5 ml final volume was ascorbic acid, 1.1×10^{-2} M. Additional reagents were maleic acid, 4×10^{-2} M; TRIS, 6×10^{-2} M; sodium diethyldithiocarbamate (DDC), 1.0×10^{-3} M; 8-hydroxy-quinoline (8-OH-Q), 1.0×10^{-3} M; NaCN, 1×10^{-3} M; and NaN_3, 5×10^{-4} M.

from soluble ascorbic oxidase found by Steinman and Dawson (28) characteristically to be inactivated during the oxidation of ascorbic acid. The wall-bound enzyme was found to be only slightly less active after a second addition of ascorbate after a previous 1-hour reaction period. The $Q_{O_2}(N)$ decreased from 3750 to 3610 and from 2070 to 1710 when prepared in water and mannitol, respectively. Extreme sensitivity of soluble ascorbic oxidase possibly may be induced by change in structure which is caused by solubilization.

In addition to displaying the properties of ascorbic oxidase, the wall-bound enzyme was not able to catalyze the oxidation of *p*-phenylenediamine or succinate, both with cytochrome c added. No activity was found under conditions in which the cytochrome system would be expected to oxidize these compounds, e.g., hypertonic conditions of homogenization and assay, pH of assay near 7, addition of adenosine-triphosphate, magnesium chloride, and cytochrome c.

It is concluded that the wall-bound enzymatic activity cannot be ascribed to cytochrome oxidase but only to ascorbic oxidase. The lack of evidence for the peripheral localization of cytochrome oxidase contrasts with Lundegårdh's suggestion. In accordance with his views it would be unexpected to find no cytochrome oxidase attached to the wall fragments and instead to find it present on the cellular debris, as alleged to result after homogenization (18). Lundegårdh's evidence for a peripheral localization of cytochrome oxidase is indirect. Much weight is given to the observation of a rapid inhibition of respiration by cyanide and an oxidation of cytochrome c in solutions bathing wheat roots (18).

SUMMARY

Ascorbic oxidase has been identified by its various properties in the homogenates of barley roots.

The retention of activity with the cell wall fragments as the pH of extraction varies, the optimal pH range of external solution for oxygen uptake by root segments supplied with ascorbic acid, and the recovery of activity after plasmolysis of the barley root cells affirm the previously reported association of ascorbic oxidase with the cell walls of various plants.

No evidence was found to suggest that the wall-bound activity was due to cytochrome oxidase.

The author expresses his thanks to Dr. E. H. Newcomb, Botany Department, University of Wisconsin, and Dr. R. N. Robertson, Senior Principal Research Officer, C.S.I.R.O. Division of Food Preservation and Transport, Plant Physiology Unit, Botany School, University of Sydney, for their helpful criticisms and suggestions in the preparation of the manuscript. In particular the author is indebted to Dr. Folke Skoog, Professor of Botany, University of Wisconsin, for his guidance and assistance during the course of the work and writing of the manuscript.

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BRIEF PAPERS

DETERMINATION OF Fe^{55} IN PLANT MATERIAL BY THE THIOCYANATE METHOD[1,2,3]

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In some work on the absorption of chemicals through the leaves and their transport through the plant, the isotope Fe^{55} was employed. The radiation of Fe^{55} consists of a 5.9 kev energy x-ray emission produced by K-electron capture and a small percentage of weak gamma rays (1). This radiation is not readily measured by an end-window Geiger-Müller counter and, therefore, we used a gas-flow counter.

In early experiments, the plant was harvested, the various parts dried separately, ground to a fine powder and aliquots of 5 to 15 mg were placed in a shallow pan made from a brass ring and cellophane tape. This was the method first used by Mitchell and Linder (3) and later by Gallup and Gustafson (2). The small quantity of plant material was not enough to give an accurate count, and when larger quantities were used, the weak x-rays were absorbed by the material itself and by the moisture layer accumulated during the period of counting. The amount of this absorption could not readily be determined. Another and even greater source of error was the decrease in counts during the period of measurement and the fact that in no two consecutive periods were the same counts obtained. This decrease has been credited to the building up of positive charges on the surface of the non-conducting sample (5). It has been found that Aquadag (a colloidal graphite preparation) prevents the building up of these charges on the counting disk (5). Accordingly, Aquadag was sprayed over the surface of the plant powder, but it was difficult to cover the surface completely without materially decreasing the counts, and its use was discontinued.

The thiocyanate method described by Piper (4) and modified for isotope determination by Rediske (5) was decided upon. In this method, the dried plant material is digested with nitric, sulfuric and perchloric acids. The iron of the digested material is next made into a ferric thiocyanate complex, which is taken up in isoamyl alcohol. An aliquot of the isoamyl alcohol is placed on an aluminum counting pan and dried.

In practice, 1 gm or less of dried tomato plant material was placed in a 125 ml Erlenmeyer flask. Fifteen ml concentrated HNO_3 and 4 ml concentrated H_2SO_4 were added. This solution was heated gently until the first violent reaction subsided, then boiled until white sulfuric acid fumes appeared and the solution was clear. If the solution turned dark, 5 ml HNO_3 and 2 ml H_2SO_4 were added and the solution was boiled again. This procedure was repeated until the solution was colorless. The whole procedure takes 2 to 2.5 hours and is done under a hood. After the solution was colorless, it was left to fume for 2 minutes with full heat, cooled slowly and 10 drops of Superoxal (30 % H_2O_2) were added. The solution was heated to fuming again for a minute, cooled and then 25 ml distilled H_2O and 5 ml 20 % HCl were added. The solution was again boiled for 5 minutes, cooled and quantitatively washed into a 50 ml volumetric flask. If the activity of the material was high, an aliquot of one-tenth the original was placed in a 50 ml volumetric flask and distilled water was added to make up to about ¾ the volume of the flask. It was found advisable to keep the acid concentration near to that of the original solution. Therefore, when only part of the original solution was used, HCl was added together with the water. It will be observed that in the above procedure, the perchloric acid included in the directions given by Piper has been omitted because higher counts were then obtained.

To the samples, prepared as outlined above, in the 50 ml flasks 2 ml 20 % potassium thiocyanate and 2 ml isoamyl alcohol were added. The stoppered flask was shaken for 20 to 30 seconds and allowed to stand until the water and alcohol separated. If the alcohol phase did not reach the narrow neck of the flask more water was added. The ferric thiocyanate complex was now in the isoamyl alcohol. It was found that if the preparation at this stage was permitted to stand for two hours before the alcohol was removed, the count was considerably higher than if pipetted off at once.

With a dry pipette 0.2 ml of the isoamyl alcohol solution was placed on an aluminum counting pan (0.3 × 3 cm). The sample pans were placed over a 1.3 cm hole in an asbestos plate held 7.5 cm above an electric hot plate. After all the sample pans were filled and placed on the asbestos plate, the heater was turned on to medium heat. This procedure was designed to prevent rapid evaporation with consequent spattering. When the plates were completely dry, they were placed directly on the heater. This heat treatment breaks down the ferric thiocyanate complex and higher counts are obtained.

In his work, Rediske (5) sprayed the sample pans with Aquadag. As mentioned before, this was done to prevent the building up of a positive charge on the counting pans because of a non-conductive sample. In the present investigation, it was found that this was unnecessary if the counting pans were dried for two minutes on a hot plate and cooled quickly immediately before counting. By this procedure, counts

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[2] Paper No. 1007, Department of Botany, University of Michigan, Ann Arbor, Michigan.
[3] This investigation has been aided financially by the Michigan Memorial-Phoenix Project of the University of Michigan.

TABLE I

MEASURED RADIOACTIVITY OF SAMPLES PREPARED BY THE THIOCYANATE TECHNIC WITH VARYING AMOUNT OF Fe^{55} AND INACTIVE IRON ADDED AS $Fe(NH_4)_2(SO_4) \cdot 6\,H_2O$

ACTIVITY OF Fe^{55} IN MC*	MG INACTIVE IRON	AVE COUNT/MIN	% CONTROL
1×10^{-5}	0	3364	..
1×10^{-6}	0	442	..
1×10^{-5}	1.425	2630	78
1×10^{-6}	1.425	308	70
1×10^{-6}	7.12	211	48

* Activity of the Fe^{55} calculated by the dilution ratio from the originally supplied solution.

made on the same material over several weeks checked very closely.

It must be recognized that under any circumstance, there is some loss of activity during the process of extraction. This was brought out in an experiment in which the activity before and after extraction was compared. When 0.1 ml of a 10^{-4} mc/ml solution was evaporated on the counting pan, the count per minute was 11,444, but after the same volume of the same solution had gone through the extraction process, the count, as obtained by the evaporation of the isoamyl alcohol, was only 3,364. In a second determination with 0.1 ml of 10^{-5} mc/ml solution the counts per minute were 1,918 without and 442 with extraction, or a recovery of only 29 and 23 %, respectively.

By the nature of the method, all of the iron present in the plant material was collected in the isoamyl alcohol extract. What effect this may have on the final results was considered early. If there is an interference from normal iron, then the accuracy would be decreased and comparisons of different parts made more difficult. Experiments were set up in which known amounts of inactive iron were added to the active iron during the extraction process. Table I gives these results. It will be seen that the presence of inactive iron materially decreases the measured activity only when the amount of inactive iron becomes quite high, as it may sometimes be in roots.

A further study of the influence of inactive iron was made by adding a known amount of radioactive iron to a known amount of dry plant material which was then ashed and extracted in the usual way. In some of these experiments, the activity of the iron was held constant and the amount of dry plant material varied or different parts of the plant used. Tables II and III give these results.

TABLE II

INFLUENCE OF DRIED TOMATO PLANT MATERIAL ON THE MEASUREMENT OF Fe^{55}

ORGAN USED	MG DRY MATERIAL	COUNTS/MIN	% RECOVERY
0	0	4400	..
Leaves, upper	500 *	2840	65
Leaves, lower	500	3380	77
Stems	500	3030	69
Roots	500 *	1870	43

One ml of a solution of 10^{-5} mc/ml activity was added to the dried material obtained from different parts of the plant. The usual ashing-extraction procedure was followed.

* The amount of inactive iron in the leaves and stem was about 0.1 mg and in the roots about 1.0 mg per 500 mg dry plant material.

TABLE III

INFLUENCE OF DRIED LEAF MATERIAL ON THE MEASUREMENT OF Fe^{55}

MG DRY WT OF LEAVES	COUNTS/MIN
0	4400
250	3970
500	2360
1000	2000

One ml of a solution of 10^{-5} mc/ml activity was added to the dried tomato leaf material. The usual ashing-extracting method was followed.

Inactive iron whether added as inorganic or as plant material decreases the measurable activity of Fe^{55}. In plant work, one can make direct comparisons between stems and leaves within a plant, but that cannot be done with roots, which contain considerably more iron than the other organs of the plant. Comparisons among different species of plants or even of the same species if grown under different conditions should not be made until an analysis has been made of the inactive iron in the plants under investigation.

That inactive iron is not the only substance interfering with the determination of active iron becomes evident when one compares tables I and II. In table I it is shown that 1.425 mg of inactive iron decreases the recovery by about 26 %, but in table II with leaves containing only 0.1 mg iron in the plant material the decrease in recovery was approximately the same as with the higher iron content. This added interference of the plant material could be due to some other ash element also going into solution in the isoamyl alcohol. In table IV we have worked out correction factors which can be applied to the data.

The small quantity of Fe^{55} that can be measured may be illustrated by an experiment. When 500 mg of root material was used, 100 counts per minute were recorded. If this value is multiplied by 6 (see table IV) to correct for the interference of the inactive iron

TABLE IV

CORRECTION FACTORS FOR CALCULATING Fe^{55} IN PLANT MATERIAL FROM THE RADIOACTIVITY WHEN 1×10^{-5} MC Fe^{55} WAS USED

TREATMENT	COUNTS/MIN	CORRECTION FACTOR
Evaporated on counting pan	11,444	..
Fe^{55} added to 500 mg dry leaves and extracted	2,840	4.0
Fe^{55} added to 500 mg dry roots and extracted	1,870	6.1

and the method of extraction, and by two to correct for the 50 % loss due to the geometry of the counter, we get 1200 disintegrations per minute. This is equal to 5.4×10^{-7} mc, since 1 mc = 2.22×10^{9} disintegrations per minute. Inserting the specific activity (797 mc/gm) of the Fe^{55} used, the 5.4×10^{-7} mc becomes equivalent to 6.8×10^{-7} mg Fe^{55} as the amount in our sample of 500 mg of dried root material. This quantity could not be measured by chemical methods.

Summary

A method has been presented whereby dried plant material is wet ashed and the contained radioactive Fe^{55} converted to a ferric thiocyanate complex, which is then taken up in isoamyl alcohol. After evaporation of aliquots of the isoamyl alcohol on sample pans the count is made in a gas-flow counter.

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TRANSLOCATION FROM SOYBEAN LEAVES. II [1,2,3]

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By the feeding of radiocarbon dioxide to individual soybean leaves, the rates of movement of specific products of photosynthesis have been determined under a variety of conditions (1). This communication presents a method whereby radiocarbon dioxide may be fed to localized areas of a leaflet, rather than the leaflet or leaf as a whole. The results of some of the initial experiments performed with this apparatus are presented.

The apparatus used for the generation and administration of radiocarbon dioxide is shown in figure 1. The mercury in the flexible, translucent tubing (e.g., Tygon) may be adjusted in height to accommodate any volume of carbon dioxide by raising or lowering the funnel end of the tube. Barium carbonate-C^{14} ($\sim$ 1 mg) is placed on the mercury surface and the end of the tubing capped with a serum vial stopper. Approximately 0.5 ml of 10 % perchloric acid is added by means of a hypodermic needle and syringe inserted through the stopper. The carbon dioxide which results is withdrawn by a clean needle and syringe, the mercury rising as the gas is withdrawn. By further lowering of the mercury and by insertion of the needle with the syringe partially filled with air, the residual carbon dioxide may be expanded further and a second withdrawal made. In this manner essentially all of the gas generated may be withdrawn.

Previous to the preparation of the gas, an L-shaped cylinder (fig 1) is adjusted to the natural position of the leaf and to that area which is to absorb the gas. The angle of the cylinder is directed downward to catch any spray resulting from the gas injection. The flange of the cylinder is greased lightly and pressed gently onto the leaf immediately prior to the addition of $C^{14}O_2$ into the cylinder.

Following the experiment, the plant is radioautographed in the usual manner within an x-ray cassette, the latter being placed in a freezing chamber. If reautographed after thawing, the xylem, originally devoid of radioactivity, is now filled. The semipermeability of the phloem is obviously lost by freezing.

A typical radiogram resulting from the localized feeding of radiocarbon dioxide to a soybean leaflet for 20 min at 200 fc is shown in figure 2.

By means of this very simple technique it becomes possible to perform a variety of experiments related to the phenomena responsible for translocation. Thus, by shading part of a leaf, one may readily determine the extent of movement of the photosynthate from the illuminated to the dark areas. As the result of at least triplicate experiments, generally involving 20 min of photosynthesis with $C^{14}O_2$, the following qualitative results have been obtained.

1. Under normal circumstances, flow of photosynthate is greatest to the growing regions. No other physiological condition approaches this as a causative agent for the direction and magnitude of translocation.

2. No appreciable flow (as shown by the lack of radioactivity outside the area fed $C^{14}O_2$) occurs within a leaflet or to an adjacent leaflet merely because of a sugar concentration gradient. Numerous experiments have been performed in which ½ to ¾ of a leaflet or an opposite leaflet has been kept in the dark 48 hours prior to the feeding of a normal adjacent area or leaflet. The effect of starvation is never greater than the normal variation of leaf intake.

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[2] This work was supported in part by contract with the U. S. Atomic Energy Commission.

[3] Contribution No. 362 of the Institute for Atomic Research.

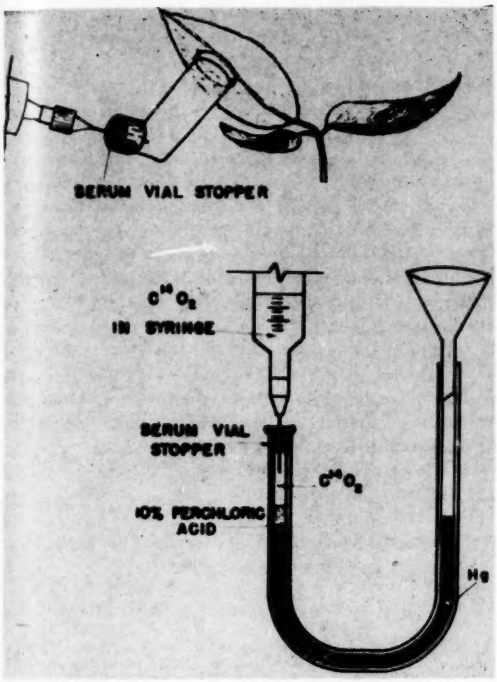

FIG. 1. Diagram of the apparatus used for the generation of radiocarbon dioxide and its feeding to localized areas of a leaf.

3. Flow will not occur out of a photosynthesizing excised leaf 24 hours after excision at the petiole. This is indicated by the absence of radioactivity in the dilute nutrient solution or distilled water in which the petiole is immersed. In most cases there is no movement whatsoever out of the area fed, not even to the rest of the leaflet. Translocation is resumed following the initiation of roots, as may be expected. The extent of plugging of the vessels as a result of excision has not been determined anatomically, but flow will not occur out of a leaflet if the petiole has been steamed locally, a fact which has been ascertained previously by a number of investigators.

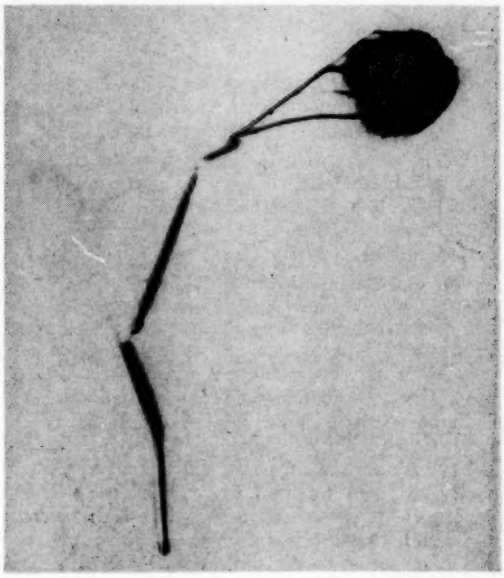

FIG. 2. Typical radioautogram resulting from the localized feeding of a soybean leaflet for 20 min at 200 fc. Note that movement out of the fed area to other parts of the leaflet and to other leaflets is essentially nonexistent. Some translocation to a lower, younger trifoliate has occurred.

4. Upward flow to a younger trifoliate leaf will occur, though diminished, even if the stem below the photosynthesizing leaf is steamed, as long as the anatomical connections of the veins remain intact.

More detailed experiments are in progress. The theoretical implications of the above and related isotopic experiments will be the subject of a future communication.

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OXIDATION AND DECARBOXYLATION OF AMINO ACIDS BY SQUASH PREPARATIONS[1,2]

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Only a moderate amount of information is presently available on the oxidation and decarboxylation of amino acids by higher plant tissues. In conjunction with other studies on amino acids, it became desirable to find out whether amino acids in plant tissues undergo degradation at rates which vary sufficiently to account for the results of Wood and Cruickshank (17) and Kemble and Macpherson (6). These investigators have shown that certain amino acids are degraded in detached leaves at rates which make it unlikely that these amino acids are available for normal protein synthesis. The dehydrogenase which catalyzes the oxidative deamination of glutamic acid has been found in many plants (2) and a tryptophan oxidase has been reported to occur in pea-seedling tissue (16). Only glutamic acid has been demonstrated to be decarboxylated by plant tissue preparations (12), although the occurrence in plant tissues of histamine, hydroxytyramine, and putrescine, the decarboxylation products respectively of histidine, dihydroxyphenylalanine, and ornithine, has been reported (10, 17).

The enzyme source in the present study was an extract of acorn squash prepared essentially by the method of Schales et al (11). The ovary wall of the squash was homogenized in a Waring blendor at 4° C in M/15 phosphate buffer of pH 5.8. The brei was filtered through muslin to remove cell wall debris, and the filtrate used without further treatment.

Decarboxylation and oxidation rates were measured manometrically at 35° C by standard techniques (13). For decarboxylation studies, the reaction system consisted of 4 ml of squash extract containing 20 mg of protein, and 1 ml of a solution containing 33 micromoles of the desired substrate. Both aerobic and anaerobic (nitrogen) conditions were employed. For studies on oxidation, the protein concentration was two times that noted above. The reaction mixture consisted of 2 ml of squash extract, 0.5 ml of a solution containing 33 micromoles of the desired substrate, 1 ml of phosphate buffer of pH 7.0, and 0.2 ml of 10 % KOH in the center well of the Warburg vessel.

In order to determine the cellular location of glutamic decarboxylase, the enzyme preparation was fractionated by centrifugation into three portions: material sedimented for 15 minutes at 10,000 × g, probably nuclear and mitochondrial (7), material sedimented for 15 minutes at 100,000 × g which is designated the microsomal fraction (5), and the supernatant from this last separation. The reaction mixture consisted of 0.5 ml of a solution which contained 33 micromoles of glutamic acid, 1 ml of phosphate buffer of pH 5.8, and 1 ml of a water solution of one of the fractions or a combination of the fractions. Measurements of decarboxylation were carried out in the manner reported above.

The decarboxylation and oxidation results obtained for a number of substrates are shown in table I. It is clear that the relative rates of decarboxylation and oxidation of this series of amino acids by the whole squash preparation vary greatly.

Glutamic acid decarboxylase is by far the most

TABLE I

RATES OF METABOLISM OF VARIOUS SUBSTRATES BY A SQUASH EXTRACT

SUBSTRATE	DECARBOXYLATION μL OF CO_2 EVOLVED/HR × MG OF SQUASH PROTEIN		OXIDATION μL OF O_2 ABSORBED/HR × MG OF SQUASH PROTEIN
	AIR	NITROGEN	AIR
α-L-Alanine	2.0	0	0.6
β-Alanine	0	0	0
α-Aminobutyric acid	0	0.2	0.5
γ-Aminobutyric acid	0	0.2	0.4
L-Arginine	2.3	0	0.5
L-Asparagine	1.9	0	0.5
L-Aspartic acid	4.0	0.2	0.2
Canavanine	0.9	...	0.2
DL-Citrulline	0	0	0.2
L-Cysteic acid	0	0.8	0.5
L-Cysteine	4.0	0	2.5
L-DOPA	0	0	0
L-Glutamic acid	19.6	15.2	0.4
L-Glutamine	6.5	0.2	0.7
Glycine	1.6	0	0
L-Histidine	0.9	0	0
Hydroxy-L-proline	3.6	0.3	0.2
L-Isoleucine	2.1	0	0.4
L-Leucine	0.9	0	0
L-Lysine	2.2	0.2	0.3
L-Methionine	1.6	0.2	0
L-Ornithine	5.2	0	0.4
L-Phenylalanine	0	0	0.3
L-Proline	1.6	...	0.3
L-Serine	0	0	0
L-Threonine	0	0	0
L-Tryptophan	0.9	0	0
L-Tyrosine	1.4	0	0
DL-Valine	0.7	0	0
α-Ketobutyric acid	3.8	...	0.5
α-Ketoglutaric acid	0	0	0.5
Glyoxylic acid	0	0	0
Glutaric acid	0	0	..
Pyruvic acid	5.6	0	0
Urea	3.3	...	0

[1] Received January 26, 1955.

[2] Report of work supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Company.

[3] Present address: Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana.

active of the decarboxylases present in the preparation. Glutamic acid is decarboxylated at a high rate under both aerobic and anaerobic conditions. The amino group appears to be necessary for decarboxylation, since α-ketoglutarate is not decarboxylated under the conditions used. The presence of glutamic acid decarboxylase in the soluble fraction of the squash preparation is evident on the basis of the data shown in table II. This finding is in contrast to those of Okunuki (9) and of Morrison (8) who were unable to find a soluble enzyme in any of a variety of plant tissues.

The decarboxylation of glutamine is somewhat peculiar in that the evidence suggests that deamidation does not take place prior to decarboxylation. If deamidation, which does not require oxygen, were to take place before decarboxylation, then one would expect glutamine to show a substantial rate of decarboxylation under anaerobic conditions. This did not occur. It seems possible that glutamine is oxidatively deaminated to α-ketoglutaramide, which may

TABLE II

INTRACELLULAR LOCATION OF GLUTAMIC ACID DECARBOXYLASE IN SQUASH

FRACTION	DECARBOXYLATION μL OF CO_2 EVOLVED/HR
Mitochondrial	5
Microsomal	5
Supernatant	211
Supernatant plus microsomal	225
Supernatant plus microsomal plus mitochondrial	228

then undergo oxidative decarboxylation to form the amide of succinic acid.

In bacterial (4) and mammalian (1) preparations amino acids are decarboxylated equally well under both aerobic and anaerobic conditions. In the squash preparation this does not occur. Instead, all the amino acids, with the exception of glutamic, are either not decarboxylated anaerobically, or show very low rates of decarboxylation under anaerobic conditions. Apparently they are preferentially oxidatively deaminated to the respective keto acids and are then decarboxylated.

The rates of oxidation by the present preparation are quite low as compared to rates obtained in similar experiments with *Neurospora crassa*, rat kidney, *Proteus vulgaris*, and cobra venom (3). The data suggest that deamination of amino acids in higher plants is largely by transamination and is not primarily an oxidative process. This is in accord with the work of Wilson et al (15) who have demonstrated both the presence of highly active transamination systems in a variety of plant tissues and a low capacity of these tissues for reductive amination. It is probable that oxidative deamination may be the limiting step in the aerobic amino acid decarboxylation illustrated in table I.

The author wishes to acknowledge the advice and assistance of Professor James Bonner and Dr. G. C. Webster during the course of this work.

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The Southern Section: The Southern Section of the American Society of Plant Physiologists met with the Association of Southern Agricultural Workers (ASAW) in Louisville, Kentucky, February 7 to 9, 1955. Two excellent sessions of submitted papers were presented with approximately 75 to 80 persons in attendance. A joint symposium entitled "Growth and Development of Plant with Special Reference to Light and Temperature" was sponsored together with the Horticultural Section, ASAW, and a second symposium, "Plant-Soil-Water Relations" had as cosponsors the Crops and Soils Divisions of the Agronomy Section, ASAW. Approximately 150 to 200 people attended the symposia. At the traditional breakfast, Dr. John W. Mitchell gave a very interesting and informative address, "Agricultural Application of Growth Regulators." Fifty people were present at the breakfast address.

Officers for 1955–56 are: Chairman, John B. Whitney, Jr., Clemson Agricultural College; Vice-chairman, Wayne C. Hall, Texas A. and M. College; Secretary-Treasurer, C. C. Wilson, University of Georgia; Directors: P. J. Kramer, Duke University; R. W. Bledsoe, Florida Agricultural Experiment Station; D. E. Davis, Alabama Polytechnic Institute; H. E. Hammar, U.S.D.A., Shreveport, Louisiana.

The 1956 Meeting will be held at Atlanta, Georgia. —Aubrey W. Naylor.

The Western Section: The Western Section of the American Society of Plant Physiologists will hold its annual meeting as usual with the Pacific Division of the A.A.A.S. The meetings will be at the California Institute of Technology in Pasadena, June 21 to 23. All physiologists are invited to attend.

The program will include four sessions of presented papers and two symposia: "Photosynthesis" and "Active Absorption by Cells."

Further information on the program and on housing and registration may be obtained by writing N. Higinbotham, Secretary, Western Section, Department of Botany, State College of Washington, Pullman, Washington.—N. Higinbotham.

HERMANN AUGUSTUS SPOEHR

Dr. H. A. Spoehr died June 21, 1954, at the age of 69, after a very short illness. He had been in his usual physical and intellectual vigor, carrying on active research, to within a week or two of his death. He had written important chapters in the history of plant physiology in America, and was recognized as a wise counselor and scientific statesman.

Spoehr was born in Chicago in 1885, attended the schools of that city, and at the age of 17 entered the great University so newly established there. He graduated in chemistry in 1906, then studied in Berlin with Emil Fisher, and in Paris the following year. He returned to Chicago to take his Ph.D. in 1909.

His first paper, on the oxidation of sugars by alkaline peroxide and iron salts, was published in 1910. He was appointed associate in chemistry in Chicago, but within the year came the call which turned him toward plant physiology. He was invited by D. T. MacDougall to join the staff of the new Desert Laboratory, just organized at Tucson, Arizona, by the Carnegie Institution of Washington. Here the young organic chemist was confronted with the difficulties which succulent and other plants face in the desert. He published a monograph on the "Carbohydrate Economy of Cacti" (1919) and another (with J. M. McGee) on "Plant Respiration and Photosynthesis" (1923).

Meanwhile, the Carnegie Institution had established its Coastal Laboratory at Carmel, California, to which Spoehr moved in 1920, becoming its Assistant Director in 1926–28. Here studies in tree growth, pigments and photosynthesis were carried out. But while Spoehr loved the dark woods, the white beaches and the looming mountains of Carmel, and long maintained a house there, the disadvantages of its isolation and separation from a university became apparent. In 1928, the year in which he became director of the Division of Plant Biology, the laboratory moved to the Stanford campus. Here Spoehr remained, except for brief interludes in New York and Washing-

ton, or foreign travel. He was an excellent administrator, and the laboratory which he designed and directed is a model of efficiency and equipment. He assembled an able and loyal staff, and welcomed many visiting investigators, so that the institution became one of the most active centers for plant research in the country.

Spoehr's own work dealt largely with carbohydrate chemistry and metabolism, on which he published many papers. But he is probably best known for his great monograph on "Photosynthesis," which appeared in 1926. This careful and critical review of a subject which had been poorly integrated previously, focused attention on the important problems, and without question stimulated the greatly increased activity in this topic for the following twenty years. Important work on pigments was done in the laboratory by Smith, Strain, MacKinney and others; and Emerson, Lewis, and Manning came later to work on photosynthesis in algae. Largely as the result of such work, Spoehr became interested in the mass culturing of Chlorella. With Milner and others he worked out the conditions of light, temperature and mineral nutrition which determined wide variations, and led to remarkable control, of the fat and protein content and yield of this alga. This inspired the active interest of the Carnegie Institution in these problems, which led to the recent monograph on "Algal Culture," which appeared about a year before Spoehr's death.

Recognition of his work was widespread. He was a member of the American Philosophical Society, Fellow of the American Academy of Arts and Sciences, and a member of several foreign societies. He was a charter member of the American Society of Plant Physiologists, and received the Charles Reid Barnes Life Membership in 1928. He was president of the Society in 1944–45. He was instrumental in the establishment of Annual Reviews, especially of Biochemistry and Plant Physiology. For one year (1930–31), he was Director of Natural Sciences in the Rockefeller Foundation, and once again administrative work took him from research, after his retirement, when he spent a year (1950) as consultant to the State Department on International Scientific Relations. This was indicative of his broad interests and sense of responsibility. He was a wide reader, and, more than most scientists, aware of the social and economic implications of science. He was a thoughtful writer and effective speaker on such matters, as those know who heard his presidential address before our society. Other occasions of this sort were presented at the annual exhibitions of the Carnegie Institution, in Washington, which he took very seriously, and worked hard to perfect.

Though he held an appointment as Professor of Chemistry (by courtesy) in Stanford, Spoehr taught but little in his long career. But he remained interested in youth, and the Spoehr home in Palo Alto always welcomed young people, especially foreign students of the University. Colleagues and visitors also found it a center of lively discussion and warm hospitality. In both of these, Florence Spoehr participated, and all Spoehr's friends have a special affection for her, and sympathy with her now.

Careful experimenter, able administrator, lucid expositor, and wise friend, Hermann Spoehr will be long remembered in the science he did so much to advance.—L. R. Blinks.

Plant Analysis and Fertilizer Problems—P. Prevot, Editor. Institut de Recherches pour le Huiles et Oleagineaux, 12 Square Petrarque, Paris 16, France. 263 pages. March, 1955. $4.00 postpaid.

This volume is a collection of papers presented at a symposium organized by the Institut de Recherches pour le Huiles et Oleagineaux and under the auspices of the 8th International Botanical Congress, Professor H. Lundegårdh, chairman. The informal discussions and debates following the presentation of each paper are also presented.

Papers dealing with some of the fundamental physiological relations of plant composition to growth and yield are presented by: H. Lundegårdh (Sweden), F. Steenbjerg (Denmark), A. Arland (E. Germany), T. Wallace (England) and O. Biddulph and A. S. Crafts (U.S.A.). A second group of papers concerning plant analysis methods are presented by: R. L. Mitchell, D. Nicholas (British Isles) and A. W. Specht, P. F. Smith and J. W. Resnicky (U.S.A.). A third group of papers is concerned with the application of plant analysis techniques to nutritional problems. Authors contributing to this group are: J. Carles, S. Trocme, J. Fr. de Ferriere, P. Halais, A. Loue, P. Prevot and M. Ollagnier, M. Ferrand and J. F. Levy (France and its colonies), L. Hershberg and R. M. Samish (Israel), C. Tamm (Sweden), W. S. Iljin (Venezuela), M. L. Salgado (Ceylon), M. Drosdoff, J. L. Haddock, W. Reuther and P. F. Smith (U.S.A.), Applications of plant analysis to such diverse crops as coconut, coffee, citrus, tung, grapes, deciduous fruits, wheat, sugar beet, sugar cane, oil palms, peanuts and others are considered.

This volume is the record of the first international symposium dealing with the fundamentals, techniques and applications of plant tissue analysis methods to problems of nutrition of plants. It is evidence of the growing body of literature on this subject, the increasing number of workers interested in this field, and the value of this approach in evaluating the mineral nutrient requirements of crop plants. No worker interested in soil fertility and mineral nutrition will want to be without this valuable group of contributions.—Walter Reuther, *U. S. Horticultural Station, Orlando, Florida.*

American Institute of Biological Sciences: As of March 15, 1955, the offices of the American Institute of Biological Sciences will be located at 2000 P Street, N.W., Washington 6, D.C. The American In-

stitute of Biological Sciences (AIBS) is a voluntary, non-profit organization of professional biological societies and individuals, that have in common an interest in the life sciences. Organized in 1948, within the framework of the National Research Council, the Institute is now assuming an independent status. Over 37,000 biologists are represented by AIBS.—AIBS.

VIROLOGY: Academic Press Inc., Publishers, announce a new journal, VIROLOGY, for which the following scientists have accepted editorial responsibility: Dr. George K. Hirst, Public Health Research Institute of the City of New York, Inc. (*Editor-in-Chief*); L. M. Black and S. E. Luria, University of Illinois, Urbana (*Editors*); C. H. Andrewes, C. A. Brandly, Seymour S. Cohen, A. H. Doermann, John F. Enders, Charles A. Evans, Werner Henle, A. D. Hershey, Francis O. Holmes, Frank L. Horsfall, Jr., Hilary Koprowski, André Lwoff, James W. Moulder, Glenn S. Pound, Theodore T. Puck, A. F. Ross, H. K. Schachman, Edward A. Steinhaus, Robley C. Williams, C. E. Yarwood (*Associate Editors*).

It is planned to publish one volume per year. Volume I, No. 1 is scheduled for release in May 1955. Subscriptions for the Volume I, priced at $9.00, should be sent to the publishers, Academic Press Inc., 125 East 23d St., New York 10, N. Y.—L. HENLEIN, *Academic Press Inc., New York.*

To avoid any misunderstanding by the members of the American Society of Plant Physiologists and as a courtesy to Professor Poúl Larsen, the following comment is published.

Four of the five members of the Committee on the Nomenclature of Growth Substances submitted a report to the Executive Committee of the Society, and to the editor of PLANT PHYSIOLOGY for publication. This report was accepted by the Executive Committee and appeared in PLANT PHYSIOLOGY, vol. 29, p. 307, in May of 1954. Dr. Larsen did not sign that report and he submitted a minority report which was not acted upon by the Executive Committee, but was submitted to the editor for publication. Since there was strong criticism to publishing a minority report, the editor took the document submitted by Dr. Larsen and changed the title with the addition of the words, "A Criticism" and rewrote the first paragraph and made certain minor changes. This was transmitted to Dr. Larsen by airmail for his concurrence. Two days before the July issue went to press, a new draft was received from Dr. Larsen differing in several particulars from the manuscript he submitted to the editor on October 26, 1953. Through an error in the editorial office, for which the editor accepts sole responsibility, the copy transmitted to the printer was the earlier copy submitted by Dr. Larsen in October 1953 with the minor editorial changes stated above, instead of the manuscript returned by Dr. Larsen in May of 1954. For this error, an apology is due Dr. Larsen. This report was published in the July issue of PLANT PHYSIOLOGY, vol. 29, p. 400, and treated as a part of the "News and Notes" section. Since this section is regarded by the publisher as the editor's responsibility, all copies of the galley came to the editor.

However, the editor had no intention of censoring Dr. Larsen's views, and, in fact, if the editor had accepted the advice of others in the Society, he would not have published Dr. Larsen's disagreement. Therefore, we are publishing below the most recent draft that we received from Dr. Larsen, unchanged, except for the correction of one misspelled word. Signed—THE EDITOR.

NOMENCLATURE OF PLANT GROWTH SUBSTANCES

In 1951 a committee of five members, including the present writer, was set up by the American Society of Plant Physiologists "to consider and propose a uniform nomenclature on growth substances." A report entitled "Nomenclature of Chemical Plant Regulators," signed by four of the members of the committee, appeared in the May, 1954, issue of Plant Physiology. The present writer did not sign the report as he was opposed to various points in the set of definitions proposed by the Majority of the committee. A brief statement of the opinion of the Minority follows.

Regulator. The word Regulator has a status in Biology as a term for the means by which living organisms maintain the harmony and balance of their various physiological processes in spite of the action of external factors. *To regulate*, according to Webster's Dictionary, means "to adjust so as to work accurately and regularly." 2,4-D and several other synthetic growth substances do not do this. 2,4-D, therefore, is not a regulator; and Regulator should not be used as a broad term including substances foreign to the organism. If a broad term for organic, oligodynamic substances, influencing a variety of physiological processes, is needed, Ergon(e) would be a suitable word; but within the field of growth processes the term Growth Substance is sufficient.

The Minority, therefore, proposed the following set of definitions.

Growth Substances are organic compounds which at low concentrations promote, inhibit, or qualitatively modify growth. Their effect does not depend on their caloric value or their content of essential elements.

Comment: On account of the ambiguity of the word "nutrient" (cf. proposal by the Majority), particularly when both autotrophic and heterotrophic organisms are considered, this term should be avoided, and is unnecessary.

Growth Hormones are growth substances which regulate growth and are produced by the organism itself. They usually move within the organism from a site of production to a site of action.

Comment: For the meaning of "regulate," see above.

Auxins are growth substances characterized by

their capacity to induce elongation in shoot cells when applied at suitable concentrations.

Auxins may, and generally do, affect other processes besides elongation, but the effect on elongation is considered critical. They are generally acids with an unsaturated, cyclic nucleus. Indole-3-acetic acid is commonly used as a standard in determining auxin activity.

Comment: In the proposal by the Majority, the following period is part of the definition of auxins: "They resemble indole-3-acetic acid in physiological action." The Minority is opposed to defining a group of substances by one of its members and to the statement that auxins "resemble" indole-3-acetic acid . . . .

Auxin Precursors are compounds which in the plant can be converted into auxins.

Anti-Auxins are compounds which inhibit competitively the action of auxins.

Growth-Inhibitors are substances which retard growth in both shoot and root cells and have no stimulatory range of concentrations.

Comment: This definition is necessitated by the preceding, specific definition of anti-auxins. A logical, systematic name for oligodynamic growth-inhibitors would be auxocholins, analogous to Köckemann's blastocholins.—Poul Larsen, *Department of Botany, University of Bergen, Bergen, Norway.*

Note to Plant Physiology

The writer wants to express his appreciation of the editor's comment on the publication of the note "Nomenclature of Plant Growth Substances." A few supplementary remarks may be permissible.

The writer had expected his minority report to be published as such. The editor, however, in his letter of May 13, 1954, proposed to publish a comment on the majority report instead. The writer consented to this. It is clear that certain alterations in the original report were necessitated by this change of procedure. The editor had proposed various modifications included in the copy accompanying his letter of May 13. The writer was unable to approve the editor's version (the one which was unintentionally printed in the July, 1954, issue of Plant Physiology), and, therefore, made such changes as he deemed necessary. The writer's revised manuscript was mailed on May 19, 1954; and its acceptance by the editor was acknowledged on May 31, 1954.—Poul Larsen.

June 25, 1955.